From Biomass Towards Chemical Ecology: Progress and New Perspectives for the Study of Food Webs in Marine Ecosystems

Thesis

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From biomass towards chemical ecology: progress and new perspectives for the study of food webs in marine ecosystems

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Abstract

Changes of paradigms in the study of food webs in the last decades summarize a fast evolution of concepts: starting from a mechanistic vision of life, scientists recently approached to the emergent properties of ecosystems and started to investigate the informational functions of foods. The development of concepts is illustrated in this thesis by means of various papers, starting from a numerical evaluation of fluxes, moving then to biomasses and energy and approaching finally to several examples of “functional foods” influencing the ecology of key species in complex ecosystems. This exercise also permits comparison of the results with a model-based vision of ecosystems and the development of a general equation linking biodiversity with the availability of trophic resources. The equation, derived from mathematical analysis of networks, is proposed to be applicable to any network of organisms or cells, at various structural levels. It is based on an exponential decay function and it has been here tested on field data obtained in such different environments as a temperate harbour, seagrass beds and coral reefs. Results are encouraging, since a good fitting of the function to the experimental data has been demonstrated.
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Section 1. General introduction
Summary of the Section

The study of food webs summarizes the fast evolution of several ecological concepts in the last decades. Starting from a mechanistic vision of life and a static concept of natural communities, it evolved through concrete steps towards the present vision of functional foods and emergent properties of ecosystems. Thus, following some ancient and modern ideas it will be shown that, starting from numbers and energy, general laws may be reached ruling communities at different organization levels – from cells to ecosystems– taking into account the abundance of trophic resources and the way they are distributed among individual consumers. Following this synthesis, the main objectives of the thesis will be outlined.
Chapter 1. History and importance of food web investigations

1.1 Historical milestones in ecological research: the genesis of ideas and their time evolution

The idea of this research grew out of several conversations with students, when it emerged that they had limited knowledge of the standard theory upon which our present ecological exploration is based. The question of whether the understanding of history is important to ecologists conducts to philosophical issues, about how theory changes actually proceed fast in ecology and which critical changes of paradigms characterized the second half of the previous century. The starting point of this change of paradigms was the need, felt in the first half of the century, to demonstrate that ecology may be considered a numerical science, with the same intrinsic dignity of mathematics and physics. In fact, previous ecological research was limited to structural characters of communities, unable to produce canonical hypotheses and test them experimentally. Thus, following the success of the International Geophysical Year (1957-1958), the British Rudolph Peters and the Italian Giuseppe Montalenti started discussing the need for an international program of biological studies focused on the productivity of biological resources and the human adaptability to environmental changes (Hillmann et al, 1989). A Planning Committee was established in 1964, proposing an International Biological Program (IBP), implemented in the wake of the successful International Geophysical Year, with G. Montalenti as the chairman. The committee recommended to be focused on the effects of environmental changes on natural communities and on the conservation and growth of natural resources for human benefits. Quite a modern topic, after all! This demonstrates how the program influenced further steps of ecological research and definitely made feeding relationships one of the core thematic frameworks in ecological sciences (Layman et al., 2015).
Accordingly, during a General Assembly held in Paris in 1964 and attended by 150 lead participants from worldwide scientific academies, a special committee was established for the IBP, for directing the program. The activities of the program ended in 1974, but the advancements it produced in our ideas are still influencing ecological research. The first critical results, however, emerged immediately in the first years of the IBP activities. In 1968 Odum published its “Energy flow in ecosystems: a historical review”, that changed the mind of thousands of young ecologists, included the writer. Here, some revolutionary (for the time) concepts were introduced, as the fact that “trophic levels are not primarily applicable to species” and that ecosystems could be analysed by means of “computer models”, simulating their activities by means of the flows of matter and energy. Fundamentally, this meant the possibility for realizing models (thanks to the first diffusion of electronic calculators), hypothesize basic relationships derived from field evidences, then test assumptions and reach conclusions that could evolve in time, just like physicists did with their theoretical systems. What a change!

The basic concepts bringing the attention of scientists on food came from far, since the concept that food is important in ecology as a limiting factor for animal populations and a regulating element for benthic communities was commonly accepted (Sousa et al., 1981; Garrity and Levings, 1983; Fairweather et al., 1984). The localized effects of predation, including grazing (Tegner and Levin, 1983; Penny and Griffiths, 1984; Thayer, 1985), were among the most extensively studied and important causes of spatial variability. As a matter of fact, the behaviours of all evolutionarily successful organisms are constrained by two major dictates (Yeakel and Dunne, 2015):

1) they must pass down their genetic material, and
2) they must acquire the necessary energy to do so.
Thus, feeding relationships have important implications for ecology, evolution and persistence of plants and animals. It has been demonstrated that even mass extinctions of the Cretaceous-Paleocene were influenced by the structure of trophic relationships and the continuous evolution of species is paralleled by an evolution of the trophic structure of ecosystems (Barnosky et al., 2011; Lotze et al., 2010). Older studies demonstrated that manipulations and experimentations could lead to concrete changes of paradigms. For example, some experiments demonstrated that specific perturbations resulted in loss of a key benthic predator, causing the community to shift to a totally different configuration (Barkay and McQuaid, 1989). Thus it was clear that comprehension and modelling of matter and energy fluxes in any ecosystem enable to comprehend its internal dynamics and built provisional models to calculate the effect of events that may modify the community structure (MacFaiden, 1964). These studies conducted to immediate results in simple ecosystems and, while computer platforms evolved, becoming more powerful, they led to interesting hypotheses in ecosystems of increasing complexity.

At the time, ecology could be defined, according to Odum (1959), as “the study of the structure and function of nature”. Energetics was concerned with energy transformations occurring within ecosystems, including feeding: a plant may be eaten by one animal that, in its turn, is eaten by another, and so on. Such a sequence of events was termed a “food chain”. However, it became progressively more evident that food and feeding relationships of plants and animals in any ecosystem are rarely, if ever, as simple as a single food chain.

For example, in the case of seagrasses, the problem was even more complex, due to the role played as both refuge for animals and food source, by such different interacting compartments as leaf stratum, rhizome layer, detritus, epiphyte layer (Irlandi and Peterson, 1991; Mazzella et al., 1992). First researches were performed on the importance of detritus feeder species in P. oceanica ecosystem (Verlaque, 1981; Tenore and Rice, 1980; Tenore, 1981; Velimirov et al., 1981). Studies were also performed to define the influence of grazers on leaves of P.
oceanica and other seagrasses (Asmus and Asmus, 1985; Klumpp and Nichols, 1983; Verlaque, 1990; Stenek, 1982). The importance of the epiphytic layer for macrozoobenthic vagile organisms, however, was initially underestimated (Verlaque and Boudouresque, 1984), although we know, now, that the total epiphytic biomass produced in a year at a shallow stand off Lacco Ameno d’Ischia (Italy) is high and accounts for 109 grams (dry weight) per square meter (Mazzella and Ott, 1984). Actually there was no information about the fate of this biomass; consequently we did not know the structure of this important path of energy transfer.

The complexity of feeding relationships in ecosystems could be illustrated taking into account the high number of producers (e.g., seagrass standing stocks, different species of epiphytic macrophytes, microphytes), first consumers (fishes, echinoderms, decapods, amphipods, polychaetes, molluscs, etc.), secondary consumers (crustaceans, echinoderms, fishes), and last consumers (fishes, large decapods) cooperating for its stability. The large number of species interacting each other produce a complex “web”, which regulates and integrates the system. Therefore it was clear that the simple food chain could not be considered as an isolated linear system; in contrast, it interconnects with a large number of other food chains. This interconnection leads to the trophic relationships of organisms expressed diagrammatically as a web. The discovery and description of a food web in any given habitat must be considered to be an enormous task (Phillipson, 1964; 1966).

Species and communities associated to various compartments of any ecosystem, their feeding mechanisms, reproductive features and rates of growth had to be analysed in the first phase of researches. This qualitative approach represented the background to the investigation of any system and led to the application of quantitative methods for estimating the abundance of common organisms and, ultimately, of the total amount of living matter or biomass. An example of such a survey is given by some older studies (e.g., Mazzella et al. 1990; Gambi et al., 1992) on Lacco Ameno d’Ischia P. oceanica bed. In addition it is interesting to
consider the foreword to the new edition of the Pimm’s Food web text (2002) because it contains an historical review of the main phases leading to the analysis of multi-species models based on population dynamics, consumption and growth rates of various elements in each compartment. The parameterization of multi-species models, made easy by the application of simple spreadsheet programs, made possible the production of predictive models based on a large portion of the literature published in the last decades. Also, the work by Pimm (2002) provides evidences for the definition of various fixed properties of food webs, based both on mathematical assumptions and field data. For example the “connectance”, i.e., the fraction of possible inter-specific links that is realized in a real web (May, 1972), depending on the number of species (biodiversity) and their trophic preferences may determine the stability of ecosystems. A related parameter, the “linkage density” appears to be as a constant feature of several ecosystems, being:

\[ L = d n \]  

Where \( L \) is the total number of trophic links; \( d \) is the linkage density; and \( n \) is the number of species, so that food webs are likely to be “scale invariant” in this and in other key properties.

On average, the number of trophic linkages was demonstrated to be about double the number of species in a range of ecosystems (Pimm et al., 1979). Very interesting is the finding that connectance declines hyperbolically with the number of species. In fact, this mathematical relationship is in accordance with our field data (still unpublished) indicating that the biodiversity is inversely related to the abundance of trophic resources, following a trend of hyperbolic decline:

\[ C = \frac{L}{s^2} \]  

\( C \) = connectance; \( L \) = total number of trophic links; \( s \) = species richness.
where $C$ is the total connectance, $L$ is the total number of trophic links and $S$ is the number of species in the system. This relationship, for values of $S>0$ brings to the trend reported in figure 1: this is what we could expect, from a simple mathematical point of view, in the representation of the connectance $C$ according to increasing values of $S$ (namely, alpha diversity).

![Figure 1: plot of the function (2) for values of $S>0$.](https://www.desmos.com/calculator)

Different methods were employed for determining food chains and food webs. In some instances, simple observations were sufficient as a first approximation. For example, given molluscs might be restricted to certain species of tunicates and it was demonstrated that many of these molluscs were eaten by a few crustaceans. However, simple observations never assured that all possible links in food webs had been considered.

A second approach was to collect representatives of all species occurring in a given system and to analyse their gut contents. Provided that a sufficient number of individuals of each species was examined, it was possible to state which animals fed upon what and, thus, build a food web (Zupo and Fresi, 1984). This type of investigations required taxonomic knowledge not only for whole organisms, but also for their separate parts. Radioactive isotopes and precipitine
tests started to be used to identify digested parts of organisms without skeletal parts. These last methods, however, were tedious and the results obtained did not always justify the amount of work performed. The simple analysis of gut contents was often considered as a good balance between research effort and quality of results.

However, a need for producing generalized representations or models of food webs emerged, even after detailed investigations had been made, as the more knowledge was available about a particular food web, the more complex it apparently became. It is possible to group together animals with similar feeding habits and similar predators, to build “ecothropic groups” (as used in Ecopath models: www.ecopath.org). Furthermore, it demonstrated to be quite difficult to compare food webs in one situation with another, because different species were involved in each case. For this purpose, feeding indices were proposed, which allowed comparisons of species and ecosystems based on their trophic features (Zupo, 1993).

The interdependence of various parts of food webs was made clear by dividing the flows into further trophic levels, starting with the primary producers which use solar energy and fix inorganic nutrients (first trophic level), passing through the herbivores (second trophic level), the carnivores (third trophic level), and reaching ultimately the decomposers (Odum, 1959; Phillipson, 1966, Fenchel, 1970). Some authors (Elton, 1927) noted that the animals at the base of a food chain were relatively abundant while those at the top were relatively few in number and there was a progressive decrease between the two extremes. This "pyramid of numbers" was found in several animal communities all over the world and the widespread occurrence of such a phenomenon provided a common denominator by which different communities could be compared. The most comprehensive form of investigations, however, was the measurement of the flow of organic matter and energy throughout each component of the ecosystem. In fact, it came clear that a simple numerical approach was not enough informative, due to the
different sizes of animals and plants. The difficulties were overcome by using the biomass of organisms rather than their number (pyramids of biomass).

Biomass is defined as the amount of living substance composing studied organisms. Alternative terms are standing crop or standing stock. The biomass of aquatic organisms is measured by weighing them after removing the excess water. A more accurate method consists in measuring the dry weight, after excluding those parts that are not living (external skeleton, calcareous parts, shells, etc.). Measurements of biomass alone, however, were quite inadequate for the purpose of predicting yields on a time basis. The rates of production of organic matter by various members of natural communities and their trophic dependence on one another or on sources outside the community had to be known. Measurements of biomass, in terms of energy released from tissues, when they are fully oxidized to water and carbon dioxide, overcame the problem of defining what are the living and non-living parts of a consumer or a prey. Furthermore, energy units were the same for all organisms. The measurements of energy contained in the tissues of organisms (pyramid of energy), gave information about the process rates (turnover time) and overcame the problems arising with the pyramids of numbers and biomass. Energy units provided a unifying concept, a means of expressing the productivity of an individual organism or all the organisms within an ecosystem. Further, the concept permitted comparisons of the productivity of regions as different as a desert and a marine environment. Energy flow refers to rates of change of biomass. The whole flow is divisible into a number of separate processes, which together indicate the passage of matter or energy throughout organisms, populations or ecosystems under investigation.

Hence, when food was finally measurable in terms of energy, computations of energy flow could be employed to assess actual and potential yields of exploitable benthic species, to predict means of increasing productivity, and to estimate the contributions made by benthic or planktonic communities to predators occupying other habitats. In addition, in order to determine the amount
of energy used by herbivores over a given period (Lubchenko and Gaines, 1981), it was important to investigate the use they made of energy. Since energy is a conservative item, energy changes between parts of an ecosystem had to be accountable by the first law of thermodynamics: Conservation of Energy.

A convenient and frequently used unit of biomass in terms of energy is the Kilogram-calory (Kcal) expressed per unit of surface. When biomass was to be measured in energy units, it was usually necessary to measure the calorific content -or heat of combustion per g dry weight- of the biological material either directly, by burning it in a bomb calorimeter, or indirectly, by determining its approximate chemical composition as proteins, carbohydrates and lipids. Known average values of calorific contents for each component were applied. The first method, which is more accurate and direct, was often applied.

Interestingly, energy mainly flows in the benthos of aquatic communities at the second and third trophic levels (previously described), which are among the easiest measurable components of an ecosystem and also the most interconnected (in terms of connectance) according to Pimm (2002). A simple method of “icons” was proposed by Odum (1959) to indicate each intermediate of the system (e.g., primary producers, consumers, etc.) and formulate simple graphical representations of ecosystems, described by energy flows within each intermediate. Furthermore, this method described the efficiency of energy transfers between trophic levels and the use of energy to overcome environmental constraints. These subjects were important not only to define basic principles underlying the functioning of ecosystems, but also to suggest methods of preservation and exploitation of natural environments (Zupo et al., 2016).
1.2. Energy as the key descriptor of ecosystems

Given the previous assumption, the research was further concentrated on energy as the main descriptor of relationships in any ecosystem. The first researches tried to answer fundamental questions as:

- What proportion of stocked energy is used to increase the overall standing crop of herbivores?
- How much is passed to carnivores?
- Is the remainder dissipated as heat of respiration?

It was necessary to assess the number, biomass, mortality, moulting losses, and respiratory rate of each main species of animals in order to answer these questions in any natural environment. For these studies the terminology of IBP (International Biological Programme) was used, according to Holme and Maclntire (1984). The appropriate IBP terms and symbols for components of energy flow for heterotrophic organisms are still quite informative:

**Production (P):** That part of the assimilated food or energy that is retained and incorporated in the biomass of the organism, but excluding the reproductive bodies released from the organism. This may also be regarded simply as "growth". It is useful to reserve the term "productivity" for the potential rate under ideal or stated conditions, and use the term "production" for the actual rate of incorporation of organic matter or energy (Davis, 1963).

**Egesta (F):** The part of the consumption that is not absorbed but is rejected as faeces;

**Absorption (Ab):** The part of the consumed energy that is not rejected as faeces;
**Excreta (U):** The part of the consumption that is absorbed and later passed out of the body as secreted materials, usually in an unwanted form as, for example, in the urine, or exudates as milk, mucus, nematocysts, etc. The combined energies of F + U are sometimes referred to as **Rejecta**;

**Assimilation (A):** That part of the consumption that is retained for physiological purposes as production (including gonoproduc ts), and respiration, but excluding rejecta;

**Respiration (R):** That part of the assimilated energy that is converted into heat, either directly or through mechanical work performed by the organism;

**Gonad output (G):** That part of the absorbed energy that is released as reproductive bodies. Because of its great importance in survival and recruitment, this part of the energy flow is separated from excreta (U) and production (P); some authors might regard it as being a contribution toward either of these elements of energy flow (Bagenal, 1978);

**Yield (Y):** The part of the production or excreta (in a varied sense) used by mankind or other predators. It may refer to only part of the organism or it may refer to a fraction of the individuals in a population, which are consumed by a predator or harvested by man;

**Mortality (M):** The mortality due to all causes, including any yield Y;

**Efficiency of assimilation:** The ratio of the food absorbed into the organism to the total amount of food ingested;

**Growth efficiency:** the total energy of production of body tissues and gonads, as a fraction of the food ingested;

**Coefficient of ecological efficiency:** the measure of the efficiency of energy transfer from one trophic level to the next. The simplest definition is the fraction of the energy consumed at a given trophic level (n) that is exploited by a predator at the next trophic level (n+1), referring to the yield to the predator as Yn.
Hence, assuming conservation of energy, we are able to set the following fundamental equations:

\[ (3) \quad C = P + R + G + U + F \]
\[ (4) \quad Ab = C - F = P + R + G + U \]
\[ (5) \quad A = P + R + G \]
\[ (6) \quad \frac{Ab}{C} = \frac{(C - F)}{C} \]
\[ (7) \quad \frac{(Y_n/C_n)}{C_n} = \frac{(C_{n+1}/C_n)}{C} \]

The equation (3) is interpreted as the fundamental equation of total energy budget and it was applied to any system where all energy sources and all energy sinks were known. This budget could be applied to a species and, with increasing difficulty and complexity, to a population or to all the organisms constituting an ecosystem. Since an energy budget must balance, should any of the terms be particularly difficult to measure, it was omitted and found by difference. However it was preferable, whenever possible, estimating all the terms of the equation, and use them as a check of the accuracy of the flow-sheet.

For some practical purposes the production term (both as primary and secondary) in the energy budget (\(P\)), was the only essential one. It was also the most important quantity to be measured in fundamental studies of ecosystems and productivity.
1.3 Secondary production measured by energy budgets: historical and methodological issues

There are two different approaches in the measurement of the total secondary production by a population, which must be clearly distinguished. The first is to take into account the growth increments of all members of the population during a given period (e.g., 1 year). The second is to take into account the fate of the biomass that has been produced during a given survey.

If the biomass is expressed in energy units, it must be conserved and the above relations remain true. If the biomass is expressed as weight, the loss of biomass due to respiration and excretion is usually not taken into account and the above equations are not applicable, since they involve R and U. The changes of biomass in the energy flow can be referred either to a given area of the system or as a fraction of the existing amount of standing crop present. Investigations tried to measure the previous constants and calculate, on the basis of cited equations, the other variables of energy flow in any ecosystem.

If \( B_0 \) is the standing crop biomass at the start of experiment and \( B_1 \) is the standing crop biomass after a given period, then:

\[
P = (B_1 - B_0) + M = dB + M \quad (8)
\]

The previous equation implies, generally, that population dynamic studies must be performed and applied to the energetic data in order to obtain a yearly standing crop and a total energy budget for an ecosystem or a given compartment. In fact, productivity measurements require knowledge of the rates of growth in terms of dry weight or energy and this information, about whole compartments of a food web, can be obtained from population dynamics (e.g., based on size-frequency histograms of monthly data).

Population estimates were normally obtained by taking random samples of fauna at time intervals (e.g., 1 month). Animals could be counted, weighed, and their energy value determined by chemical processes or by means of bomb-
calorimeters. Also the energy contained in foods and the energy residual in faecal pellets was measured. Respiration rates were measured by respirometry sets and O$_2$ electrodes.

The heat of combustion of foods, faecal pellets and animal bodies was used to calculate some of the above-mentioned variables. Heat of combustion was measured in bomb calorimeters, usually made of steel and filled with oxygen under pressure. For some biological work, a sufficient quantity of dry tissue was not easily available (e.g., faecal pellets produced by small benthic organisms) and thus, micro-bomb calorimeters were used instead. The Phillipson (1964) micro-bomb calorimeter had a capacity of 8 ml and took samples of 5-10 mg.

Faecal pellets, due to their low calorific content, burned too slowly. Since it was most important that the samples burned completely and smoothly, some improvements were normally achieved by compounding a known amount of benzoic acid of standard thermodynamic quality into the pellet. For these kinds of substances, an alternative to direct calorimetry was the use of indirect chemical methods. These approaches, however, were not sufficiently accurate for small amounts of substances.

The ingestion and egestion were difficult to measure, especially in small organisms, and there were a few satisfactory studies of energy flow applied to benthic species. Rates of ingestion and egestion were normally measured in the laboratory. There were, in principle, two types of methods available:

1) **direct methods**: the food ingested and the faeces produced by the animal, over an adequate period of time to establish continuity, were measured directly.

2) **indirect methods**: the concentration of an indigestible marker present in, or artificially incorporated into the food, was measured in both food and faeces, together with the rate of faecal production.
The simplest marker was the organic ash content of the food itself, although the method was very inaccurate and perhaps unreliable if the amount of ash present was small. Conover (1966) employed this technique for measuring the ingestion rates of copepods; it was assumed that all the ash absorbed in the food was present in the faeces, and this influenced results. However, other inorganic compounds (e.g. salts, and calcareous substances) and markers added to the food (e.g., vital dyes like Methylene blue) could be partially assimilated. Thus, the method of ash measure represented a good balance between the accuracy of results and the influence of the food quality on the organism to be tested.

Energy losses by respiration were directly measured by respirometry, although the values obtained accounted, generally, only for a fraction of the actual amount of energy to be considered as “cost of maintenance”. In fact, anaerobic respiration and heat losses contribute to this value. For general ecological purposes, the value obtained was considered accurate enough. According to Elliot & Davidson (1975) and Ivlev (1934), in view of other experimental errors involved in the measurements of energy losses through respiration, the assumption that 1 ml of oxygen at normal pressure and temperature is equivalent to 29 J, was sufficiently close for all practical purposes. The knowledge of chemical composition of foods ingested was useful to reduce the error in the conversion of oxygen uptake into energy losses. The energy lost per mg of oxygen was variable according to the elementary composition of foods and tables were available (Elliott & Davidson, 1975) to calculate oxycalorific equivalents of different compounds. On the basis of previous considerations methods were devised to calculate the energy budget in various environments.
1.4. Various types of theoretical models and their consequences

1.4.1 The shape of food webs

In general terms, food webs are networks of interactions among species, groups of organisms, populations, or aggregate trophic units (Winemiller and Polis, 1996). Different approaches were proposed by scientists, according to the features of target ecosystems, available instruments of study and aims of the research (Dunne, 2009), whether they were predictive, descriptive or simply theoretical. Yet in 1927 Elton suggested that food webs are a central topic in the field of animal ecology, although at his time food chains were considered as the basic concept in this field, presently almost deprived of importance in most complex ecosystems. At the time, simple models of trophic interactions (Bronmark, 1985) offered the most direct framework to understand how entire ecosystems functioned and several concepts relevant for food webs were emphasized, as the role of body size (Thayer et al., 1984), the pyramid of numbers, the concept of niche, and various indirect effects of food webs. Some of these topics are still interesting fields of scientific discussions, as a demonstration of the key importance of the concepts linked with food webs and interactions (Brose et al., 2006). In fact, the concepts presented by Elton, as the introduction of “pyramids of numbers”, influenced ecological sciences for decades, and most notably Lindeman (1942) and Odum (1953) ideas on ecology, while Elton (1927) initially tried to balance the search for simple theories with the recognition of ecological complexity in natural systems. This concept is recurrent in present research trends.

1.4.2. The vision of Elton

The initial vision of Elton (1927), in terms of interactions between prey and predators, evolved rapidly into the Lindeman (1942) vision of prisms of energy and nutrients: a concept that directly generated, after some time, the above
mentioned ideas of the IBP (Nicholson, 1961). Energy networks permitted the definition of a common currency for studying and comparing diverse aquatic ecosystems (Lindeman, 1942). A key advancement of his characterisation of ecosystems was the recognition of abiotic and biotic compartments through which organic matter is transformed. This vision brought to the first reference to the concept of “ecosystem”, as “the association of abiotic and biotic components” (Golley 1993): another demonstration of food webs as an underpinning concept for the ecology theory... and it continues to expand today.

1.4.3. The visions of Gardner and May

In fact, the theory further expanded through the IBP, as above referred, to reach full maturity, demonstrated that –at list in part- ecosystems may be modelled using energy networks and considering “animal characters” as energy transducers (Gardner and Ashby, 1970; Williams and Martinez, 2000). However, only after the end of the Program it was possible to foresee how the concept of “food webs” was closely interconnected with some key features of ecosystems, as their stability and diversity (May, 1972; McCann, 2000). In addition, various further evolutions of these concepts indicated interrelationships, demonstrating that biodiversity influences stability of natural communities and vice-versa (Dunne, 2006; Ives, 2005; McCann, 2000; 2005). In a mechanistic (not totally accepted) view of ecosystems, the number of nodes (system size), the number of links among nodes (system complexity), and the interaction strength among nodes influence the relative degree of stability of ecosystems (Gardner and Ashby, 1970). In fact, generating small perturbations to equilibrium values of nodes, and checking whether values return to the previous equilibriums, the stability levels of various ecosystems are assessed.
1.4.4. Emergent properties

Alternative parameters and “emergent properties” (Novak, 2006) were further proposed in the never-ending search for an easy assessment of system’s stability, as the *ascendancy* (Banerjee et al. 2017), the *biodiversity* (Bastolla et al., 2005) and the *connectance* levels (Liu et al., 2017). Various authors demonstrated other emergent properties of ecosystems (Novak, 2006) according to the structure of their food webs (May, 1972), as the inverse relationship between connectance (a measure of the food web complexity, based on the proportion of realized ecological interactions among the potential ones; Poisot and Gravel, 2014) and species richness (directly related to biodiversity, ruled by the equation (2) above mentioned). Various groups (Briand, 1983; Briand and Cohen, 1984; Cohen et al., 1981) further investigated these patterns. As above indicated, not all the conclusions of May were generally accepted and some of them are still discussed, in the light of modern theories. However, his studies revolutionized this scientific field, in terms of theory and practice, and they continue to impact and inspire investigations on food webs, especially through quantitative analyses of their structural properties. In addition, present research in evolutionary ecology still proposes individual-based stochastic models, where ecological communities are considered “emergent structures” derived from interactions between organisms.

1.4.5. The TaDa model

A very interesting and modern view of this concept is the *TaDa model* (Tangled Nature model) proposed by Christensen et al. (2002). According to this model, individuals are vectors in a genotype space and their reproductive success is determined by the fitness of each individual to his own environment. Some conclusions of this model, as the exponential extinction of the node degrees according to the density of species, will be further discussed along with my general model of species diversity related to the abundance of resources.
His theories on the effects of connectance received attention by further authors (Gardner and Ahsby, 1970) that re-defined the properties of ecosystems. The models attempted to mathematically establish their levels of stability and complexity. In particular, they modulated the number of nodes (system size), the average number of links among nodes (system complexity), and the strength of interactions (effects of strength of nodes).

The first pure theorization of ecosystem properties led to an useful process of hypothesis and test, considered to be a milestone in the study of food webs and, more generally, in ecology. For example it was demonstrated that structural properties of networks affect the stability of ecosystems and, in particular, an increase in the number of nodes, complexity, and interaction strength represents a destabilization factor for natural networks. Also, researches by May and his followers demonstrated that if the interaction strength in a stable system is kept constant while species richness is increased, the system might remain stable only if the overall connectance decreases. Thus, an inverse relationship between connectance and species richness was demonstrated. This assumption was further investigated and obtained subsequent support from investigations on natural food webs (Briand, 1983; Cohen et al., 1985; Pimm, 1982; Yodzis, 1980). We will use these relationships to propose a general model linking species richness to the abundance of trophic resources.

1.4.6. Measuring web properties

As above specified, the theorization of specific properties of food webs primed further analyses based on hypotheses and these focused on specific metrics measuring the web properties, as the measures of connectance, food chain length, degree of omnivory, proportion of species at given trophic levels, population densities, etc. (Pimm, 1979, 1982, 1984). Going deeper in the results of these analyses would be redundant here, but it is worth to observe that, very simply, the introduction of ecological realism in mathematical models (e.g., non-random trophic structure imposed to the system), implies that predictions on food
web stability diverge from results based on randomly assembled mathematical models. This evidence primed researches in next years (e.g. Cohen, 1978) on real-world food webs aiming at predicting their structure based on quantitative metrics. One of the most interesting theories, in this view, was the so called “cascade model”, based on just two variables represented by:

a) species richness
b) linkage density (total number of links divided by species richness).

The hypothesized system had two constraints only:

1) species are randomly assigned to a one-dimensional feeding hierarchy;
2) species can only feed on lower elements in the established hierarchy (Cohen and Newman, 1985).

This very simple model, based on two basic assumptions, was effective in reproducing the structure of food webs in a range of ecosystems, indicating that similar underlying structure could rule several ecosystems.

Next advancements of food web theory indicated that a network model could answer several questions about the stability and functionality of ecosystems and allow for a simulation of food web dynamics (Dunne et al., 2002a; 2013; Thompson et al., 2012). Similar to small world networks, investigations indicated that food webs tend to have short path lengths (Williams et al., 2002), while deviation from scale-free structures in networks indicated that food webs should be more robust to species extinction than other forms of networks, as a result of high levels of connectance. In contrast, food webs with lower connectance are more sensitive to species extinctions. For example, it was demonstrated (Dunne et al., 2002b) that extinctions are more likely to propagate throughout networks with abundance of trophic specialists.

1.4.7. The conclusions by Winemiller

However, empirical data used to set and test food webs were insufficient to depict real-world feeding relationships and in often underestimated the actual strength of species interactions (Winemiller and Layman, 2005), making the
prediction somewhat weaker and uncertain, from a mathematical point of view. First examinations of tropical basin’s food webs demonstrated quite higher number of interactions and here it has been demonstrated, for the first time, a central concept that will be expanded throughout the present thesis: the completeness and the resolution of simulations is a basic attribute to obtain clear demonstrations of food web properties (Winemiller, 1990; Winemiller, 1989; Winemiller and Polis, 1996; Winemiller et al., 2007). However, in-deep investigations require huge amounts of research work. For example, Winemiller (1990) examined about 4800 guts in each tropical watercourse to obtain sufficient conclusions on the degree of connectance in his empirical research. Actually, most investigations on food webs took into account only a limited number of nodes/animal groups, often from a single point of view (e.g., without considering the actual surface area or the volume where species lived), using different metrics, sometimes taking into account “suspected” but unverified feeding relationships. This led to easy generalizations and simplifications about the actual complexity of ecosystems (Cohen et al., 1993). This concept will be further used to propose a general model for evaluation of actual resources available for food web characters (the so called RAFL Index; Zupo et al., 2017). Actually, the spatial and temporal boundaries of food webs are arbitrary and each author investigated subsets of feeding interactions in a given region, generally chosen for practical reasons (a seagrass meadow, a portion of coral reef, etc.), not considering that ecosystems are interconnected and any boundary is an (indispensable) artefact influencing the conclusions of any study. Another important corollary deriving from the researches by Winemiller (1990) was the demonstration of a positive relationship between connectance and species richness, such that more diverse webs had higher connectance. This conclusion derived from quantitative analyses, that contrasted prior assumptions (Briand, 1983; Pimm, 1982), and indicated an inverse relationship between connectance and species richness. The discussion continued up to the present,
with opposite visions. Hence the concept is worth to be investigated, because from it may depend our comprehension of life expansion and biodiversity. The investigations contained in this thesis partially diverge from those of Winemiller and it will be demonstrated, based on empirical data and mathematical simulations, that species richness is inversely related to the abundance of resources. This is the fundamental concept that will be expanded within this thesis to reach higher assumptions and demonstrations on the consistent properties characterizing assemblages of natural life, extensible to communities at various organizational level, from single cells to the largest ecosystems (Shoener, 1986; 1989).

**1.4.8. The classical vision of Polis**

However, prior to conclude the general presentation of the history of food webs it is important to consider some fundamental studies conducted mainly on sub-aerial systems, whose conclusions may be extensible to marine ecosystems, claiming for the need of direct assessment of real-world webs (Polis, 1991; Polis and Hurd, 1995; 1996; Polis et al., 1997; Polis et al., 2004). For the first time the interactions among ecosystems (e.g., how terrestrial inputs affect ecosystem functions in freshwater ecosystems, or the impacts of marine productions on terrestrial food webs) were measured and quantified, demonstrating that the theoretical elimination of habitat boundaries brings to a better understanding of food web dynamics and to concepts important to investigate the landscape ecology. Probably, due to the need of generalizations to afford food web studies (as above referred, we need to hypothesize hypothetical boundaries, reduce the number of connections due to lack of information, underestimate the flows of detritus, etc.), further studies tended to approach the problem from an alternative heuristic point of view, generalizing empirical information to compile energy flow through webs. For example a modern frame of research uses stable isotopes and molecular descriptors, in order to define time- and space-integrated properties of
food webs, avoiding the “snapshot of recently ingested prey items” provided by classical gut content analyses (Layman et al., 2012; Hobson and Welch, 1992). In fact, stable isotopes provide time-integrated estimates of trophic positions avoiding the need for extensive diet analyses performed on individual consumers (Vander-Zanden and Rasmussen, 1999). Stable isotope analyses are surely useful to produce information on bioaccumulation and help understanding the fate of trophic resources within the pathways of food webs. However, they do not allow detailed modelling of fluxes and of resource partitioning (van Valen, 1965), as previous studies did, and this makes unfeasible the evolution of mathematically formulated theories on the relationships between biodiversity, connectivity and abundance of resources.

For example, it has been recognized that individuals within a population vary according to their trophic roles (Roughgarden, 1972; Schoener, 1986). The importance of this concept was maximized by studies demonstrating that some “generalist” taxa are actually composed of several individuals each characterized by a specialized diet (Bolnick et al., 2003). Investigations on the “ecology of individuals”, in fact, led to newer assumptions, but also complicated our ability to model real-world food webs, in respect to the above mentioned stable isotope studies. In addition, they are only partially important to discuss the thesis herein contained. Thus this theory will not be afforded in deep, here. A single important point determined by this theory is that individuals of the same species may exhibit high overlap in resources use at low population densities, but at a higher conspecific density individuals will diverge in trophic ecology to minimize intraspecific competition (Bolnick et al., 2011).
1.4.9. Synthesis of fundamental approaches

On the whole, all the above-mentioned researches indicate two fundamental approaches to the problem of food webs:

a) webs of energy flows and

b) functional webs (Paine, 1980).

For example, it was demonstrated that the experimental removal of the starfish *Pisaster ochraceus* (Asteriidae, Forcipulatida) resulted in a local decrease of species richness, because the population size of a dominant competitor, a bivalve mollusc, increased excluding lower competitors (Paine, 1966, 1969a, 1969b, 1974). Thus, again, a significant effect was pinpointed, indicating a relationship between structure of food webs, abundance of trophic resources and biodiversity of the target community. In particular, an increase of the target “food” for a starfish (i.e., bivalve molluscs) was translated into a decrease of biodiversity for that specific trophic level. In addition, this theoretical advancement also led to the establishment of the concept of “keystone species”, in this case referred to the starfish, strong and highly influential for local food web dynamics.

In parallel, the dynamics of trophic cascades indicated that nutrient inputs determine the productivity of a system and cascading effects have broad implications for understanding food web dynamics and ecosystem functions (Carpenter et al., 1985; 1987; 2001).

1.4.10. Moving towards non-trophic interactions

Since this step, the attention of scientists was further attracted towards new horizons, represented by non-trophic interactions. In fact, the food web structure is definitely a function of the total sum of direct feeding interactions between consumers and their resources, but indirect effects, like density-mediated indirect interactions or allochemical and infochemical substances, may tune the efficiency
of trophic cascades (Schmitz, 1998; Schmitz et al., 2000; Schmitz et al., 2004; Werner and Peacor, 2003). These initial observations opened the way to the idea of “functional foods” and to the “emergent” (Novak, 2006) properties of food webs (anticipated by the TaDa model previously introduced), to explain how direct feeding interactions are insufficient to completely explain the complexity of food webs, and why behaviour, chemical interactions and life cycles, often co-evolved between plants and animals living in the same ecosystem, may be critical to understand the structure of food webs and the temporal dynamics of trophic fluxes.

Finally, as above referred, co-evolution of species (Post and Palkovacs, 2009) and evolutionary biology, in general, may be crucial to understand contemporary developments and relationships among mathematical and structural properties of food webs (Schoener, 2011). As a consequence, the attention of food web investigators definitely moved from simple observations of their structure to an experimental approach aiming at the comprehension of emergent properties, as those revealed by chemical interactions among species. In addition, the above considerations demonstrate how food webs have implications in species, population, community and ecosystem dynamics, involving mathematical modelling, physiology studies and chemical relationships, up to co-evolutionary dynamics. Thus, in the last decades scientists approached the study of food webs and debated about the right scientific perspectives according to divergent and continuously changing viewpoints, as indicated, alternating empirical and theoretical approaches.

1.4.11. Food webs and biodiversity

The scopes of this thesis go beyond the aforementioned points of view since it aims at identifying general patterns of food webs, to be applicable to a large range of plant and animal associations, even at various organisational and structural levels, as some previous authors attempted (Riede et al., 2011; Layman et al., 2015). For this reason, the next chapters of introduction will summarize the
work of various authors addressing the interactions between biodiversity and resource availability, in order to detect fundamental mathematical relationships to support the main hypothesis. These relationships will be further tested, according to the model for rapid detection of trophic resources to reach the conclusions of the thesis.

However, prior to start the description of the time evolution of the notion of food webs in the last decades, it is important to define key premises indispensable to transform the next chapters into a hypothesis to be tested and validated on field data to answer the main question of this thesis. To this end, some mathematical relationships of networks will be introduced in order to show how complex ecological patterns may be modelled into simple equations, whose validation will represent the main output of the thesis.
1.5. Evolution of concepts and the need for a “model”

In the next chapters examples of the evolution of the concept of food webs in the last decades will be provided. Several studies performed on this topic exhibited a “hope” for a synthetic model able to reproduce the complex patterns of energy and matter circulation in nature. Scientific research moved towards multiple aims, individually important to understand key processes; however, they were insufficient to find a general law consistently linking the availability of resources to the biodiversity measured in natural environments. In fact, part of the research explored the possibility to describe key mechanisms and processes, as the strength of trophic links, the patterns of presence of different types of predators and prey and the distribution of species among trophic levels (Zupo, 1993). Another sector of the research explored emergent (Novak, 2006) properties of food webs, as the changes over time of trophic relationships within complex and stable ecosystems and the role of information, often overcoming the importance of energetic relationships (Zupo, 1993; Zupo, 2000): this produced a critical shift from the sciences of energy to the concept of emergy. Finally, an important output of recent researches was the discovery of common features characterizing the food webs of such different environments as tropical reefs and temperate seagrass meadows (Zupo and Mazzocchi, 1988). Thanks to this discovery we can finally model, with good degree of approximation, the actual abundance of trophic resources available for various groups of consumers and this opens up new chances to investigate the relationships between biodiversity and trophic resource availability (Zupo et al., 2016).

Due to the lack of punctual and comparable data on the availability of trophic resources, most studies linking biodiversity and food webs investigated mainly local effects of biodiversity on the stability of ecosystems and the total biomass of consumers reached (May, 1972). In contrast, studies relating the effect of trophic resource availability on the biodiversity of consumers are almost absent because the abundance of trophic resources has been constantly investigated using a range of methods, adopting variable measures and was based on a range of
volumes or area units to evaluate stability and complexity of ecosystems (May, 1973). All this made generally impossible the comparisons of biodiversity and resource availability in different environments, to test hypotheses derived from mathematical models (Rosenzweig et al., 1999). In addition, the newest chances offered by modern computers revive the challenge to obtain complex models simulating the actual properties of natural ecosystems (May, 1999) in order to forecast their development according to global changes. However, taking advantage of the information contained in further chapters and crossing them with the most advanced modelling techniques, an attempt will be made here to find general equations and trends explaining the effects of trophic resource availability on the biodiversity of the communities of their consumers.
1.6. Modelistic approaches to trophic interactions

It is demonstrated that many species of animals directly interfere with each other through chemical, physical or other interactions (Mazzella et al., 1991), but besides these, the food web theory shows that community dynamics and structure are mainly controlled by trophic interactions (Rossberg et al., 2005). We will further analyse the consequences of these types of interactions on the levels of biodiversity of various ecosystems and a search for common laws ruling different environments will be performed, even at very different structural levels. Most researches aimed at understanding the extent to which biodiversity is important in the functioning of ecosystems and, in order to quantify this effect, investigations were concentrated on empirical relationship between biodiversity and ecosystem efficiency to perform given functions, directly or indirectly relevant to their services supporting human activities (Rossberg et al., 2006). Investigations and mathematical models often demonstrated a close relationship between biodiversity vs. biomass and productivity of ecosystems; these relationships were also related with the stability of ecosystems (e.g., Millennium Ecosystem Assessment; Hassan et al., 2005).

Thus, mathematical models were developed to describe and forecast food web functions and properties although, according to Box (1979), “models are all wrong but some of them are useful”. Many different useful models of complex systems (including food webs) exist. A conceptual reduction of ecological communities to “food webs” is commonly considered acceptable (Jennings et al., 2002) and any area of science may invest in theoretical research aimed at constructing useful ecological models and improving them according to experimental evidences. Very simple and effective models, as the Lotka-Volterra (Lotka, 1922; 1956) simulation for competition, may exemplify scenarios of community assembly using elegant simplifications, as:

$$\frac{dB_i}{dt} = (s_j - \sum_k C_{jk} B_k) B_j$$  \hspace{1cm} (9)
where $B_j$ is the biomass of a predator species, $s_j$ is the size of its population, $C$ is the consumption according to the biomass $B$ of its prey and we impose $s_j$ and $C_{jk} \geq 0$ for all $j, k$.

This model is a generalization of the logistic growth model, where several competing species hamper each other’s growth. In addition, periodic invasion-fitness distributions may complicate the model and permit evaluations of the trends of biodiversity over time. Natural quantitative limits exist to keep sustainable the species richness of a food web. Thus, species richness must be a consequence of intrinsic processes operating within food webs, not controlled by the influence of speciation, invasion, etc. However, to produce an effective model, we should also consider invasion processes, possibly leading to the extirpation of other species from a given community. Previous papers (Zupo et al., 2015) demonstrated that the community is chemically defended (Sieburth and Conover, 1965) against the attack of invader species (competition avoidance), because the community owns knowledge shared among the residents- that invaders have not incorporated in their strategy yet- about the toxicity of some prey. However, even when saturated and chemically protected, communities are not closed to invasions and an extirpation may eventually be due to a direct knock-out of a resident species by an invader or it may result from more complex interactions within the community, indirectly facilitating the process of extirpation (Yackulic et al., 2014). Thus, the natural turnover of communities also results from a continuous process of alien invasions and resident defences, leading to progressive (slow) changes of species composition, including possible changes of species diversity. Due to climate change influences, further and faster invasions of alien species support the functioning of communities. It has been demonstrated (Maibam et al., 2015) that environmental modifications due to climate changes may influence the chemical relationships among organisms and increase the vulnerability of the chemical defences protecting natural associations (Zupo et al., 2015), so facilitating an easier admission for invader species. Fixed mathematical limits constrain the number of species naturally assembled in a community, even
if species composition was progressively modified by climate changes. Interestingly, the biodiversity has space constraints: since there is less space at higher than at lower latitudes, less species may be predicted to globally co-exist, as the planet warms up and oceans acidify (Descombes et al., 2015; Zupo et al., 2015).

1.7. Essential mathematical simulations: noticeable relationships

1.7.1 How species diversity influences the community biomass

Mathematical simulations permit to evaluate the relationships between total biomass of the community \( B_{\text{tot}} \) and species diversity \( S \) according to Wilson et al. (2003) and Wilson and Lundberg (2004), using approximation techniques, as the mean-field approximation, often reported by physics. According to the mean-field approximation we can forecast:

\[
B_{\text{tot}} = \frac{SK}{1+(S-1)C}
\]  

(10)

where \( I \) represents the invasion rates and \( C \) represents the competition rates, which indicates a continuous increase of species richness, up to a plateau, normally expected in any community, until the rate of competitive exclusions of species is balanced against the rate of invasions.

This trend proceeds in time in accordance with increments of biomass, as reported in the Figure 2 (Hooper et al., 2005) and this simulation represents, indeed, an indirect demonstration that a higher diversity \( S \) corresponds to a higher biomass \( B_{\text{tot}} \).

In fact, the maximum value of \( B_{\text{tot}} \) obtained at saturation must correspond to the value obtained by the mean-field approximation for infinite values of \( S \). Thus, again, the increase of species richness is naturally correlated to an increase of biomass of the community, up to a plateau imposed by space constraints,
confirming the assumptions of several authors (May, 1972; Gardner and Ashby, 1970; Hooper et al., 2005).

![Figure 2. Relationship of $B_{tot}$ and $S$ along time, according to Hooper et al. (2005)](image)

![Figure 3. Known relationships (right) and hypothesis of this thesis (left)](image)
1.7.2 How community biomass influences species diversity

Unfortunately, the relationships described in the previous chapter about the effects of biodiversity on the biomass of resources does not add much information about the main question above stated (Figure 3), that in contrast aims at understanding:

*Does the relative abundance of trophic resource influence (positively or negatively) $S$?*

As a matter of fact, most theories tend to provide measures of biomass in order to detect the effects of biodiversity on the productivity of ecosystems and evaluate their services. An opposite theoretical approach is proposed in the present study, to forecast the diversity of an ecosystem (or part of it) according to the abundance and availability of trophic resources. In this case, should we expect that a higher availability of resources triggers a higher diversity of species, as instinctively one could forecast? Will a large amount of available plant biomass trigger an explosion of species diversity for vegetarians? Does a high abundance of organic detritus sustain a large diversity of detritivores? The answer is not trivial, since a higher availability of “prey” could theoretically both induce an increase of species number, or an increase of (larger) individuals deriving from a few species. In both cases, a good efficiency of energy transfer between levels is assured, but the effects in terms of biodiversity are quite different. This question also imposes to establish if an increase of trophic resources available for given categories of consumers will lead to a different type of interference competition, exploitation competition, or apparent competition. Interference competition is commonly associated with direct (somewhat aggressive) interactions between organisms; exploitation competition (often included under indirect competition as well) is associated with the exploitation of biotic or abiotic resources; apparent competition (sometimes called indirect competition) is associated with the resistance of two competitors to mortality by a consumer, parasite or pathogen.
they share. A numerical increase and/or a biomass increase of a few species feeding on a given resource will produce low competition and increase of the productivity. In contrast, an increase of diversity of species feeding on the same resource will lead to an increase of competition and relatively low productivity of their populations (MacArthur, 1969; MacArthur and Levins, 1964).

In both cases (increase of individuals of a few species or increase of the total number of species “S”) a large availability of feeding sources could produce a higher biomass of consumers, but the effects on the general levels of diversity might be contrasting (MacArthur, 1955). As an alternative, a higher abundance of feeding sources could theoretically have no effects on the biomass of consumers, because it could be only partially consumed (e.g., chemically defended resources) and the theory of feeding interactions may be applied to various kinds of feeding interactions (Maibam et al., 2014). Finally, various competition strategies may be theorized for producers (allochemical relationships, competition for nutrients and light, etc.) and again we should be able to predict if an increase of resources (e.g., inorganic nutrients), sufficient to trigger an increased productivity, will also facilitate variations in the biodiversity of primary producers and eventually, in which direction (positive or negative effect). May a trend be forecasted, according to the theory of networks and the data deriving from empirical studies on ecosystems? Inorganic fertilization and increase of light irradiance will lead to a simple increase of production for the plants already present (increased exploitation of shared resources), or conduct to an increase/decrease of biodiversity of plants (Makarieva et al., 2008) sharing the same resource, according to an increase of resource-mediated competition?
1.8. Mathematical networks and their properties

1.8.1. Predictable effects of production and competition

To obtain indications about this central question, we will start now by examining some fundamental mathematical properties of networks (Case and Gilpin, 1974) and we will compare them with empirical data obtained in ecological communities. For example, we do know that consumer-mediated competition is strong when producers exhibit a high carrying capacity (K) and are aggressively consumed by species efficient in the foraging upon their resources. In contrast, consumer-mediated competitive exclusion decreases when producers exhibit low carrying capacity and/or are eaten by inefficient consumers. If the abundance of consumers is constrained by internal competition, they will not have strong effects over productive communities.

Different theories were developed to address this problem. They all imply an upper limit $S_{lim}$ of the number of competing species $S$, over which the coexistence becomes increasingly difficult (Bastolla et al., 2005). Species that efficiently exploit their resources keep the abundances of producers low (Chesson, 1994). For this reason, small abundances of available resources are normally recorded, even if they are intensively produced at high rates (May, 1972). Less aggressive consumers tend to be less influential on the population size of their prey (top-down effect). However, the actual issue herein raised (bottom-up effect) is if a larger abundance of “potential” feeding sources (i.e., theoretically present in the community in the absence of their consumers) may trigger positive or negative variations of species richness $S$ within the pool of their competitive consumers (Bastolla et al., 2005).
1.8.2. Species abundance distributions

A property of ecological communities, fundamental to develop several models, is the distribution of numerical abundances of species (namely, the Species Abundance Distribution, SAD). The leading concept is that although a natural community may be dominated by a few species, we normally detect the presence of several less common and rare species. SAD is low-demanding with respect to criteria for properties and in fact McGill et al. (2007) reviewed 27 different models to reproduce and explain empirical SADs, concluding that most of them perfectly reproduced the field data, even though the ecological mechanisms considered were quite different. Sometimes we define the “complexity of a system” as the possibility to be described by a variety of different models. In this view, food webs are complex, because they fall into this category, being multi-dimensional entities. However, according to Rossberg (2012), while being guided by mathematical techniques because theory-driven ecology goes one step further, we need in-depth knowledge and data of empirical systems, but much theoretical work is just a matter of trial and error. In particular, community food webs classically describe networks of flows of energy and biomass between populations of various species, as resulting from feeding interactions.

1.8.3. Networks

From a mathematical point of view, food webs are networks because consumers (generally) do not feed on all species present in a community. This concept is quite clear and easy from a formal point of view, but it is scarcely defined and precise when we observe the actual rules in nature. In fact, a species might consume a large number of “potential” resources (several potential links) but most of the energy reaching its population could derive from a limited number of preys (main preys, due to active choice or seasonal availability of resources). This concept is difficult to be synthesized into a general theory because “theoretical flexibility” is hardly needed. This brings to various generalizations, simplifications and types of models containing large or small numbers of nodes for the
description of the same ecosystem. Different types of models may take into account the chemical composition of bodies, the spatial structure of food-webs or specific features of networks, as the connectance or the seasonal variability, the trophic transfer efficiency between levels and the number of trophic levels. For example, Lindeman (1942) proposed a relationship for trophic transfer between two next levels as follows:

\[
\text{Transfer Efficiency} = \tau = \frac{c_T}{c_A}
\]  

(11)

where \( \tau \) is the ratio of production rates at adjacent trophic levels, \( c_T \) is the constant of respiration and \( c_A \) is the assimilation efficiency for a given species. According to this equation, it is interesting to observe that the energy content of farmed salmons and the transfer efficiency of their commercial feeds, described by Tyedmers (2000), exhibiting transfer efficiencies between 15% and 19%, was in total agreement with the data reported by Brett (1986) for wild populations of salmons and their natural foods.

As expected, the proportional changes in abundance (availability, biomass, and population size) resulting from bottom-up effects, increase at each trophic level. A small increase or decrease in primary production, for example, triggers large increases or decreases of populations at higher trophic levels. The ecological reason, sustained also by the above reported equation, is that a decreased resource abundance induces less biomass being available for consumers, at each level, crossed by the assimilation efficiency, leading to a proportional decrease in consumer abundances (Rossberg, 2012). A corollary of this rule is that small perturbations of a food chain (in given top-down or bottom-up effects) might be combined, without affecting each other. From this perspective, some aspects of the historical debate of top-down vs. bottom-up control in ecosystems may appear scarcely effective to describe ecosystems (Gross et al., 2004).

Thus the “rates” each model provides are often referred to different kinds and dimensions of physical quantities. Sometimes they indicate a flow rate (having
dimension Mass/Time); elsewhere they indicate such proportional rates as the transfer production efficiency (Brey, 2001), according to the complexity of living organisms and various multi-factorial aspects of food webs. However, all the above concepts may help defining some fundamental, consistent or usual features of food webs. For example, Lindeman (1942) proposed a standard value for transfer efficiency in marine food webs, that is about 0.1 at each trophic level, and Pauly (1996) confirmed this value in various cases, although large variations are observed according to the considered ecosystems.

1.8.4. Species richness and resource availability

The number of species present in an ecosystem is normally considered as the species richness “S” and it is only partially related to the number of trophic links “L”. The population dynamics of a species may be modelled as:

$$\frac{dn(t)}{dt} = rN(t) \quad (12)$$

where n(t) is the number of individuals (population size) and r is a reproduction constant (growth rate of the population size). The size of a population, for a given species, is (approximately) proportional to its reproductive value and it is, in its turn, related to the body mass of individuals (Rossberg, 2012), which also depends on the availability of trophic resources and the rates of predation and competition. The value of r is positive when individuals of a given population reproduce faster than they die. In addition, the above equation establishes that the larger the population size N(t), the faster it increases over time. The equation has a possible solution represented by:

$$N(t) = N_0 \exp(rt) \quad (13)$$

where $N_0$ is an arbitrary constant that equals the population size at the time $t = 0$. The population becomes very large rapidly due to the exponential function
(Sugihara, 1980). Its effects on plant and animal associations will quickly become stronger and the population will drastically modify its environment. Thus the conditions that determine the population growth rate $r$ will change due to the depletion of the resources necessary for reproduction, reaching a plateau. The rates of reproduction and death depend on the composition (in terms of sex, age and genotype) of the population of $N_0$ individuals, on the health of individuals and their spatial distribution. If, on the other hand, the population growth rate $r$ is negative, the population size $N(t)$ will sooner or later reach just a few individuals, and subsequently the species will extinct after stochastic demographic fluctuations in $N(t)$.

1.8.5. Allee effects and alien invasions

The rates of reproduction might be limited by the probability of finding mates in a species with sexual reproduction, present in a given population at a low abundance. Consequently, $r$ will decrease with $N(t)$ (a process called dispensatory dynamics or Allee effect). Besides this, several other mechanisms may impact life cycles and species populations can be disrupted at low abundances, thus leading to “Allee effects” (Berec et al., 2007). Since many communities are regularly invaded by new species (White et al., 2006), the Allee effect does not represent a critical reason for population disruption at low abundance. A noticeable mechanism for overcoming such limits in the life cycles of species is the invasion of habitats by alien species along fronts. Before invading a given front, the new species cannot be considered as an invader, but after the front the populations are above the threshold of the Allee effect. Dispersion of individuals around the front can help overcoming the threshold. Such a front may move forward and the invasion proceeds when the Allee threshold is lower than half of the system carrying capacity for the invading species (Lewis and Kareiva, 1993). However, in our treatment of data to demonstrate relationships between resource availability and biodiversity (Chesson, 2000), we will not consider spatial effects; thus, Allee
effects (including issues of mate finding) will not be taken into account either, for simplicity.

1.9. **Biodiversity and common laws of functioning**

1.9.1. **Complex models**

The maintenance of biodiversity implies the maintenance of individual populations within a community. Several mathematical models calculate the theoretical survival of a given population or groups of species over time, depending also on their food availability and abundance. However, we would need to calculate the survival of each life stage (larvae, juveniles, adults, etc.) over the timeline, also according to reproduction cycles and seasons, for each species present in a given ecosystem, and finally the biodiversity of the studied environment. These complex computations may lead to frequent errors, especially when repeated for several species (de Roos and Persson, 2013; Blanes et al. 1998) since the size of populations of each species (in each stage) depends on the population size of every other species. These relationships, instead of being based on numbers of individuals, may be modelled based on total biomass (or energy units) of species and their populations, but the formal complexity of simulations remains, even using mathematical simplifications as the *Quasi Normal Approximation* techniques (QNA; Rossberg and Farnsworth, 2011).

1.9.2. **Modelling individual features**

Due to the increasing complexity of models, overcoming the performances of even larger computers, several authors found easier and more useful to abandon the goal of obtaining such detailed models of simulation and heuristically concentrate on single features of food webs, even starting from empirical sets of data, to find common laws of functioning. For example, the depletion of resources according to the species consumption may be modelled.
The time variation of a trophic resource according to the presence of a species of consumers may be modelled as (Rossberg, 2013):

\[
f(x(t), y(t)) = \frac{v^t L(x(t), y(t))w}{vTw} = 0.46 \, y^{-1} \, x(t) + 0.54 \, y^{-1} \, y(t) - 1 \, yr^{-1}
\]  

(14)

This equation was computed as a model for the resource-dependent rate of change of the population size of \textit{Rana catesbeiana} as a whole. The coefficients 0.46 yr\(^{-1}\) and 0.54 yr\(^{-1}\) characterized the strengths of resulting trophic interactions. This relationship leads to the patterns of abundance or \textit{R. catesbeiana} reported in Figure 4:

Another key parameter to be taken into account for modelling the size of an animal population is its reproductive value \(r\). For several species, including fish and marine invertebrates, the reproductive value roughly equals their body mass (Rossberg, 2012). In addition, the realized community structure and dynamics may be influenced (or even be a direct consequence) of the strength of trophic links. Trophic link strengths are “dyadic” data (Kenny et al., 2006) because they depend on the phenotypes of two species, \textit{i.e.}, the resource and the consumer species. They are both described by the trophic traits of species (\textit{e.g.}, the combination of the vulnerability and the foraging traits of each species or, in other words, their descriptions as both resources and consumers). The functional response of a consumer population (Stenek and Watling, 1982) is represented by the rate at which consumers remove the biomass of their resources through foraging,
depending on the actual availability of the resource (Rossberg, 2012). The functional response of a population of consumers may be modelled as the sum of their functional responses to their resources, multiplied by an assimilation efficiency that is approximately evaluated to be between 0.1 and 0.6 (Jeschke et al., 2002). We should consider, in parallel, that consumers tend to eat much more of the most abundant resources, according to the prey-switching model. Thus, a faster depletion of the most abundant resources should be forecasted, because all possible consumers will tend to switch their choices towards them. Notwithstanding all the above-mentioned relationships, however, we should consider that in stable ecosystems, at the equilibrium, the sums of body masses in a population (i.e., the population biomass for each species) should not change, independently from the initial population structure.
1.10. Dietary diversity and proportions

1.10.1 Basic indices of diet partitioning

Several indices of dietary diversity have been proposed (Ulanowicz and Wolff, 1991; Bersier et al., 2002). The diet partitioning function (DPF) for example depends (Quince et al., 2005) on the dimensionality of niche space and a power law with exponent \(-0.74\) (named dietary partitioning exponent) and it has been proposed to describe the sizes of diet proportions (Quince et al., 2005). Interestingly, several quite different food webs exhibited similar DPF exponents. Here it is worth to consider also the Gini-Simpson index of dietary diversity (dd), which is defined as:

\[
Gini - Simpson \text{ dd} = 1 - \sum_j p_j^2
\]  

(15)

where \(p_j\) (ranging from 1 to 0) represents the proportional contribution of species \(j\) to the total biomass of a community.

Often an index \(Z_{C_p}\) is computed to evaluate the DPF of a community, by counting all links \(l\) contributing proportions of at least \(p\) to the diet if some consumers and dividing by the number of consumers:

\[
Z_{C_p} = \frac{l}{sc}
\]  

(16)

Where \(Z\) is the number of links \(l\) divided by the number of species in a food web. Consequently, \(Z_{C_p}\) is the number of links above the threshold \(p\), divided by the number of consumers \(c\) in a food web. The Diet Partitioning Function \(Z_{C_p}\) is a function of a threshold value with \(0 < p < 1\) and it is defined as the number of food items contributing at least a biomass proportion \(p\) to the diet of a consumer, averaged over all consumers in a community (Berlow et al., 2004).
Figure 5 (From Rossberg, 2013). Index $Z_{C(p)}$ evaluated for increasing values of $D_{eff}$ of 1, 1.6, 4.6, 19, 3 respectively and reported over logarithmic axes.

The DPF largely depends on the niche-space dimensionality and diet efficiency indices, as indicated in Figure 5. From this computation we conclude that the number of links contributing proportions of biomass $Z_{C(p)}$ to consumers, is characterized by a logarithmic decay shape for the DPF, when related to the abundance of diet proportions $r$ (Connolly et al., 2005) and according to the same equation, we can expect that the biomass $B_j$ of resource $j$ is lower if its trophic link strength $A_{jk}$ with consumer $k$ is large:

$$p_{jk} = \frac{A_{jk}B_j}{\sum_{l=1}^{L} A_{lk} \theta_l} \quad (17)$$

Differently, the diet ratio $r_{jk}$ is defined as the ratio between the contribution of the species $j$ and the contributions of all other resources to the diet of the consumer $k$. Thus, diet proportions derive from a relationship that can be evaluated as:

$$r_{jk} = \frac{p_{jk}}{\sum_{l \neq j} p_{lk}} = \frac{p_{jk}}{(\sum_{l=1}^{L} p_{lk}) - p_{jk}} = \frac{p_{jk}}{1 - p_{jk}} \quad (18)$$

However, inter-specific competition plays an essential role in ecological relationships since, in the context of community ecology as well as in the frame of the global evolution on the planet, interactions between two species may produce an increase in the population of the first, that is in its turn detrimental to the second, and vice versa. Contemporaneously, several features of the
population, as its mortality, consumption and growth rate, follow an allometric scaling law in the form:

$$am^{3/4} \Delta t$$  \hspace{1cm} (19)

where a > 0 is a constant. This means that the amount of body mass $m$ gained in a short time $t$ is $am^{3/4}t$.

### 1.10.2 Relationships of resources vs. consumers

Here it is also worth to consider that the number of links connecting one node to others may be called “degree” and the degree distribution theory may explain other features of food webs. Since food web topologies are graphs with indication of flow directions, we can distinguish the “in-degree” as the number of food-species resources and the “out-degree” as the number of consumers. A mathematical analysis of this niche model indicates that it generates distinctive distributions of in- and out-degrees according to the distribution of scaled numbers of resources $k/Z$ (Camacho et al., 2002a; b). Following to this analysis, the probability that a species has $m$ consumers is the following:

$$P[m \text{ consumers}] = \frac{1}{22} \int_0^{22} \frac{t^m e^{-t}}{m!} \, dt$$  \hspace{1cm} (20)

and contemporaneously, the probability to reach $k$ resources is:

$$P[k \text{ resources}] = \frac{1}{2} E_1\left(\frac{k}{22}\right)$$  \hspace{1cm} (21)

From this also derives the integral exponential function that we will encounter in various models for the evaluation of resources in the form:

$$E_1(x) = \int_x^\infty \frac{e^{-t}}{t} \, dt$$  \hspace{1cm} (22)
All the previous are quite general mathematical laws of models, since the distribution of scaled number of resources \( k/Z \) is independent of model parameters. The same applies to the scaled number of consumers \( m/Z \) (if \( Z \) is not too small). Thus, the corresponding universal cumulative distribution functions shown in Figure 6 can be explained taking into account that the cumulative distribution of out-degree is approximately triangular, while that of the in-degree has a fatter tail, analogous to the shape of an inverse exponential function.

Figure 6. Universal cumulative distribution functions for consumers and their resources in any environment follow an exponential decay pattern (From Camacho, 2002).
1.10.3. A starting point to model biodiversity according to the abundance of resources

For the same reason, the following relationship has been proposed (Chamacho et al., 2002b; Stouffer et al.; 2007) to predict the shape of the functions explaining the probability to increase the number of trophic links (cumulative distributions) and the number of resources, as well (and very interestingly for our hypothesis!) as the diversity of consumers and the abundance of resources:

\[ \text{Cumulative distributions} = \exp(-x) - xE_1(x) \quad (23) \]

with \( x = k/(2Z) \).

According to the above relationship, the number of trophic links, that is closely related to the diversity \( S \) of consumers, depends on the abundance of resources according to the \( \exp(-x) \), as a possible answer to our main question. This is then the model we will test, through the indices RAFI (Zupo et al., 2017), and validate on trophic data collected in various environments, in the next sections. Following these two trends, we could approximately hypothesize that:

\[ S = K \times \exp(-R) \quad (24) \]

Where \( S \) is the number of species (alpha diversity), \( R \) is the abundance of resources and \( K \) is a constant of the considered ecosystem (depending on genetic diversity, turnover rates, temperature, etc.). In this case, the expected trends for the levels of biodiversity of consumers, expressed according to the abundance of their resources, should follow a typical exponential decay pattern (Figure 7).
Figure 7: study of the generic function $S = K^* \exp^{(R)}$ according to the above considerations on the cumulative distributions and their relationship with the diversity of consumers.

A question clearly arises (Rossberg, 2007): what is the underlying mechanism for these apparently universal trends? The equation above might simply be a consequence of the lower-triangular structure of food webs (Figure 8) and in fact the same out-degree distribution applies to cascade models. Since links connecting in the niche model a given species to its consumers are statistically independent and they have equal probability of occurrence (except for the triangularity constraints), this approximate independence is maintained in nature (Rossberg et al., 2006b), evidently.

Figure 8: matching-model food webs underlying the simulated distributions of in- and out-degrees according to (Rossberg et al., 2006b).
Similar conclusions may be reached when a variant of the niche model is considered Guill (2010). Thus, there is not only evidence for phylogenetic patterns in food webs, but also indications that (at least according to the principle that in most cases “the larger eats a smaller one”) phylogenetic correlations dominate the processes structuring food-web topologies.

However, and most importantly, the mathematical nature of these geometric distributions helps assuming that they may be applicable to a range of systems at various structural levels, from whole ecosystems down to the physiology of individual multi-cellular organisms, where phylogenetic and size constraints meet the triangular shape of prey-predator distributions according to the abundance of resources (Milo et al., 2002; Arim and Marquet, 2004; Bascompte and Meliàn, 2005; Stouffer et al., 2007; Kondoh, 2008). Ecological communities are dynamical systems, just like assemblages of cells within organized tissues, and this explains why we expect that the diversity of (consumer) forms observed along a range of resources may be reproduced at any organizational level with similar patterns of distributions derived by the mathematical forecasts for such networks.

A confirmation comes from the analysis of networks using the coefficient of variation of scaled abundance, expressing CV as a coefficient of variation of trophic resources. In this case, according to Rozdilsky and Stone (2001) we can expect (Vandermeer, 1970) a distribution shaped as the one in the figure 9.

This is interesting because once again it indicates that species richness in an ecosystem increases according to a quasi-exponential function, with CV. Thus, an increase of species richness contributes an exponential increase of CV up to a plateau (limit for species richness), due to competitive interactions among species. The relationship demonstrates once again that S may be related to the abundance and distribution of the trophic resources.
Figure 9. A numerical test of competition mean-field theory performed according to Rozdilsky and Stone (2001) indicating the patterns of variation of the coefficient of variation of scaled abundance of resources according to species richness.
1.11. Expected relationships with trophic level

Several studies took into account the abundance of trophic resources along what is named a “trophic level”, although this is a generalization useful for scientists since it has not an actual realization in nature, given the network structure of food webs (Zupo, 1994). However, while analysing trophic levels, we can attempt some predictions useful to address our main question, as above stated. In fact, we do know that the abundance of resources changes at different trophic levels. In particular, when we move to higher trophic levels the abundance of resources decreases according to the efficiency of conversion of prey biomass. Thus, examining the changes in biodiversity over increasing trophic levels will provide another evidence about the possible effects of prey biomass (expressed as well as number of individuals or in energy units) on the diversity of consumers.

For example, the distribution of species over trophic levels was investigated by Petchey et al. (2004) in UK. Although in their study the diversity of producers is probably underestimated, since some taxa were excluded to improve data comparability, they demonstrated a regression of species richness against levels with good agreement of the regression slope with a ratio to the richness of 1/3 as expected for multi-level food webs (Figure 10).

![Figure 10: Species richness by trophic level in freshwater ecosystems (from Petchey et al., 2004). Circles, squares, and triangles are geometric averages of data sets. The solid line is a linear regression of log $S_l$. The dash-dotted line indicates the theoretical slope.](image-url)
In a similar study, the ratio between the richness of level-2 and level-3 of consumers in British and North American freshwaters were computed (Jeffries and Lawton, 1985). They reported a high correlation, and a mean richness ratio of 0.36 with a tendency for lower values in communities characterized by higher S. Given the quantitative agreement between richness ratios observed in several types of natural communities and those predicted using random-matrix models, and observing the apparent absence of alternative explanations, we can now summarize the implications that theory of random-matrix competition has for the interpretation of community structure. These implications follow from key assumptions made in developing the theory itself. In fact, according to these simulations, at higher trophic levels (lower theoretical availability of biomass of food sources) a lower S is recorded. If this is true, it represents another demonstration of the expectation, herein proposed, that higher biomass available for consumers should be related to lower diversity of their communities. And given the above mentioned concepts about allometric scaling laws and slopes of about 1/3 of the regressions “species richness vs. trophic levels” (directly related with gross resource availability) we should expect that regressing S against the relative abundance of trophic resources in various systems, a pattern of exponential decay with negative exponent close to 1/3 should be observed. In addition, given the premises, we should expect as well that this pattern will be recorded at any structural level of an analysed systems, from the physiology of individual organisms up to complex ecosystems.

In fact, several characters of naturally complex food webs may be reconstructed starting from purely mathematical models, as the so-called “PDMM” (population-dynamical matching model, Rossberg et al. 2008). Recent evolutions of the PDMM (Fung et al., 2013; Shephard et al., 2012) combine switching functional responses and a multi-dimensional trophic-niche space, with simple Euclidean geometry and allometric scaling laws for biological rates. It was called “matching model” because of the matching of vulnerability traits with foraging traits indispensable for the establishment of strong trophic links. The model provides
not only the community structure and its internal relationships, but also insights about size, biomass and diversity of various organismic groups. The theory explains these patterns on the assumption (not always justified) that the mechanisms underlying the process could operate not only in the simplified models studied, but also at higher levels. In some cases, in fact, the PDMM answers questions regarding general relationships between community dynamics, structural stability, productivity, exploitation and biodiversity, and dependencies of these on size or trophic level.

However, in general terms we may state that two mechanisms regulate biodiversity in food webs, and they are both associated with competition: the first is resource-mediated and the second is consumer-mediated (resembling the top-down and bottom-up mechanisms often considered in the past). Both mechanisms limit biodiversity through constraints to the feasibility (rather than the stability) of equilibrium communities. The reason for this evidence is that distributions of trophic link strengths in food webs have large spreads, while variations in species biomasses appear not important. Both mechanisms naturally co-occur in models and both play the role of determining species richness at a given trophic level. Resource-mediated competition in food webs with multiple trophic levels restricts richness in one trophic level to a fraction of the richness at the lower trophic level. This fraction is approximately 1/2 in food webs with only two trophic levels, while it is 1/3 in food webs with many trophic levels: this brings back to the concept of the exponential functions exhibiting this ratio along a trend of decrease of resources. The reason why the richness ratio in multi-trophic food webs is as small as 1/3 is that resource overlap matrices are scarce and random, so that even with a comparatively small number of competing consumers, strong competitive interactions are possible. On the other end, when resources are very abundant and consequently, consumers may easily invade communities, the resulting consumer-mediated competition may still limit and regulate the richness of resource.
1.12. Expected relationships with size of organisms

The above mentioned laws also have implications on the size of organisms, when the considered resources are not (totally) protected by chemical or physical defences and they are thus regularly consumed to increase the biomass $C$ of associated communities of consumers. In fact, if the first hypothesis above-stated was true (no effect on the number of species $S$ feeding on them) then we should expect that an increase of biomass is due to either an increase of the number of individuals of consumers, or an increase of their body mass, or both. The same applies if there is an effect on $S$ and it is negative (increased biomass $Q$ of resources paired with decrease of $S$). Also in this case, in fact, if $Q$ increases and it is regularly consumed, producing an increase of $C$, then we should hypothesize either (or both) an increase in the number of individuals $N$ or an increase of individual size $Bc$.

From a modelistic point of view, a characterization of the community size structure (Blanco et al., 1994) makes use of $\beta \geq m$, defined as the biomass of living individuals with body mass larger or equal to $m$. The “size spectrum” of an aquatic community may be defined as the value of $\beta (m)$ (Platt and Denman, 1977; Rodriguez and Mullin, 1986):

$$\beta (m) = -\frac{d\beta \geq m}{d \ln m} = -\frac{md\beta \geq m}{dm}$$  \hspace{1cm} (25)

Naturally, each species has a specific size spectrum (from juveniles to adults) and the average size spectrum may vary seasonally. However, if the above mentioned relationships result to be true (exponential decay of the number of species according to increases of biomass of $Q$) then we should expect that communities feeding on abundant food sources are composed by a large number of (larger?) consumers, while communities feeding on scarcely abundant food sources should be composed by lower number of (smaller?) consumers, to assure conservation of mass.
The theory developed here gives an answer to the question of what ultimately determines absolute species richness. Previous analyses indicated that resource-mediated competition links the richness at one trophic level to richness at the next lower level. At the lowest trophic level another mechanism must be active and this could be consumer-mediated competition via the second trophic level. In various communities a top-bottom mechanism was demonstrated (Fuhrman, 1999; Thingstad, 1998) and in this case the biodiversity must be due to consumer-mediated competition.

![Figure 11](image)

Figure 11. Trophic levels of species against rank in order of falling values (From Rossberg, 2013). Dotted lines correspond to integer levels, the dash lines has a slope as predicted by the above reported equations.

Low-level biodiversity is required to sustain the species richness at higher trophic levels in natural communities. According to this theory, species richness naturally declines along with trophic levels, and usually also with species sizes. These evidences should be taken into account when quantifying biodiversity, e.g. by measuring species richness in each trophic level or size class (Figure 11).
Section 2. Aims and Objectives
Chapter 2.1. *Thesis aims (expected results)*

All the reported relationships tend towards a clear forecast, *i.e.*, the diversity of consumers *S* should exponentially decrease according to the increase in the abundance of resources *R* and it could follow a typical exponential decrease pattern (equation 24) or, alternatively, it may be viewed in terms of connectance and in this case it should follow a trend as that reported in equation (2). In both cases, for positive values of resources a typical exponential decay pattern should be recorded.

If the relationship between resource abundance and *S* in a given ecosystem will be straight, we could even consider a linear trend and in this case, given the above mentioned concepts about allometric scaling laws, a negative slope of about 1/3 of the regression “species richness vs. trophic resource availability” should be estimated.

These predictions might sound somewhat atypical, since one could instinctively expect that an increase of resources leads to an increase of biodiversity of consumers. However, as demonstrated in the previous chapters and although the reverse relationship has been well demonstrated (*i.e.*, a high biodiversity of the community leads to its increased productivity) there are clear indications that this trend should be expected. In the next chapters some investigations will be reported to trace the discoveries of various features of benthic and planktonic ecosystems, and the above mentioned hypothesis will be tested and validated on field data.
Chapter 2.2 Objectives

This thesis has two main objectives, both equally important:

(1) To illustrate, from an historical perspective, the evolution of the science of food webs, from mechanistic approaches to the modern vision of emergent properties of ecosystems, based on researches published in the last 30 years.

(2) To propose, based on recent research, a general equation of life, able to evaluate and forecast the biodiversity of organism’s associations at various structural levels, from cells to ecosystems.

To this end, some published papers have been combined to follow the theoretical evolution of investigations on food webs, moving from numerical descriptions (cpt. 2.1), trophic guilds and energetics (cpt. 2.2), and modelistic approaches (cpt. 2.3). Some key findings on the information delivered by functional foods are reported (cpt. 2.4) to exemplify the shift toward a modern vision of food webs, and highlight the importance of chemical ecology relationships among benthic organisms. This concept is deepened (cpt. 2.5) by means of some recent papers, demonstrating that food may represent information, and this step is fundamental to understand the actual functioning of natural communities associated with Posidonia oceanica meadows. In this case, wound-activated compounds trigger vital chemotactic reactions in invertebrate grazers, demonstrating that information may be as important as energy, in ecology. Finally, to reach the objective (2), the results of recent publications on a model of availability of trophic resources are reported (cpt. 2.6), because this is an indispensable step to obtain comparable trophic data on a range of ecosystems, and compare them with the biodiversity levels measured in different environments. Thus, starting from established mathematical relationships between diversity of consumers and abundance of their trophic resources, the general shape of biodiversity trends has been hypothesized and formalized into two general “equations of life”. The first equation describes the levels of biodiversity expressed according to the abundance of trophic resources, while the
second equation describes size and abundance of consumers according to the levels of food availability. The results of the proposed general equations have been validated using the model reported in cpt. 2.6 and compared with field data. They permit to forecast the trophic structure of any ecosystem and the results could be extended to other networks of live structures at various hierarchical levels (from cells to ecosystems), but further experiments will explore this hypothesis and eventually extend the validity of the two equations to a wider range of biological systems.

Thus, in the experimental chapters (Section 3), it will be indicated how:

1) The structure of food webs may be assessed based on numerical analyses and semi-quantitative data on gut contents (Chapter 3.1). Some papers have been considered that investigated the food webs of *Posidonia oceanica* and they demonstrated how, starting from numerical analyses of prey frequencies, it has been possible to extrapolate some key pathways of flow from the plant to higher predators. These relationships represent the pavements for further improvements of the theory.

2) The use of feeding guilds and energetics may provide a synthetic representation of the main functions in any ecosystem (Chapter 3.2). Since most studies in that period were taking into account feeding groups and a large scientific debate was alive about the possibility to agree on homogeneous group, to avoid an heuristic approach that led to the assignment of same species to different groups, due to personal opinions of scientists. For this reason, a new index has been proposed to classify species in homogeneous trophic groups. The introduction of the index is representative of an attempt to conduct “opinions” on food webs towards a quantitative analysis of fluxes and paths.

3) Primary production studies and investigations on plant-animal interactions started to indicate the importance of “information” within the structural properties of food webs (Chapter 3.3). This has been a critical stage in the history of food webs. The strong mechanistic convictions that led studies
up to this stage started to appear weaker when compared to the value of informational relationships among organisms. This step is important to highlight the value of information within the food webs of coastal complex ecosystems, which will be further demonstrated in the next chapter.

4) The above-mentioned topic will be further explored with newer techniques, demonstrating through the example of a shrimp co-evolved with a benthic diatom, how chemical interactions determine the ecosystem functioning (Chapters 3.4 – 3.5). The case of *Hippolyte inermis* and his diatom food, in fact, is emblematic to demonstrate that food may represent mainly a source of information, more than simply playing the role of “fuel” for consumers. In fact, this proterandric herbivoruous opportunistic species may find plenty of food in the epiphytic layer of *P. oceanica*, but specific diatoms of the genus *Cocconeis* are indispensable to “tell” information about the right time to change the sex and the presence of possible predators. More recently (Zupo et al., submitted) the infochemicals produced by *Cocconeis* spp. have also been demonstrated to be a chemical cue for *H. inermis* larvae, to detect the right substrates for settlement.

5) Finally, a model for rapid prediction of the available trophic resources was proposed (Chapter 3.6) and, using mathematical predictions derived from simple analyses of webs, tested on empirical data on food webs, it will be demonstrated that biodiversity is inversely related to the abundance of resources and that this law is valid for any system, at any level of complexity.
Section 3. Experimental \textit{(published papers)}
Chapter 3.1 Synthesis of methods used

3.1.1 Published papers

This thesis on published papers involves a range of methods used over time to reach various types of food web representations. The pathways of energy transfer numerically described in Cpt. 1 are obtained by collecting benthic organisms in seagrass meadows, using a trawl trained by a boat, and counting the organisms present in their gut contents identified, after dissection, up to the lowest possible taxonomic levels. Multifactorial statistical analyses were applied to obtain trophic structures of ecosystems. Trophic guilds described in Cpt. 2 use a similar methodological approach, and the research is based on the techniques developed in Cpt. 1, but new statistical tools have been developed in order to obtain comparable data in different ecosystems. To this end, preys were classified according to their size and type (either plant or animal). In contrast, the modelistic approach used for the researches described in Cpt. 3 involves field experimentations, because the leaves of *Posidonia oceanica* have been mechanically marked and monthly sampled to evaluate their growth rhythms, while various physical properties of their environments have been recorded. A further statistical treatment permitted to identify the main factors involved in the growth rates of individual leaves and the time patterns of leaf growth and decay. The results were used to build a mathematical model (Buia et al, 1992), to predict the primary production in various conditions and in any season, as a base to predict the circulation of energy in a seagrass ecosystem. The research described in Cpt. 4, in its turn, takes into account the life cycle of a benthic shrimp, exemplifying the importance of diatom food as a metabolic regulator. In this case shrimps were collected by divers over fixed surfaces of a seagrass by means of an airlift, identified, measured and counted prior to determine their sex based on the size and shape of the appendices on their second pleopods, dissected and observed under the optical microscopy. The techniques described in Cpt. 1 and 2 are applied here to detect the effects of individual gut contents on the process of
sex reversal. In Cpt. 5 a typical approach of chemical ecology was applied. Volatile organic compounds (VOCs) were extracted from benthic algae (epiphytes of *P. oceanica*) typically fed by benthic invertebrates (as demonstrated in Cpt. 1, 2 and 3). VOCs were concentrated by loop-stripping and incorporated into agarose blocks, further proposed to target invertebrates (collected over leaves of *P. oceanica*) to record their chemotactic reactions. Finally, in Cpt. 6 a model of trophic compartmentalization is presented, applicable to virtually any marine ecosystem and based on literature data. In this case, a large data-base has been built and filled with the abundance of trophic resources characterizing various environments, as described in previous chapters. This permitted to establish a specific index of relative abundance of food items (RAFI) useful to predict the availability of trophic sources for various categories of consumers. The RAFI index takes into account the trophic categories identified in Cpt. 2 and it is the base for the validation of the “equations of life” developed in this thesis.

**3.1.2 Statistical treatment of data in this thesis**

In order to reproduce the structure of food webs in a range of ecosystems and quantitatively predict the relationships existing between abundance of resources and biodiversity of plant and animal communities, various models of trophic resource partitioning have been examined, according to network theories. Interestingly, various models and geometric distributions, as the Universal Cumulative Distribution functions for consumers and resources, follow an exponential decay pattern (Camacho et al., 2002b). According to this trend, it has been hypothesized that the number of species *S* present in a given trophic compartment is inversely related to the abundance of their trophic resources (R) according to such an exponential decay function.

To demonstrate this hypothesis, the relationships between theoretical food availability (RAFI) and the species diversity according to feeding guilds were compared in various environments and in particular, in 13 stations distributed into the bay of Naples and in additional 41 world sites. Final scores indicating the
Relative Abundance of Food Items (RAFI%) for each of the considered environments have been obtained taking advantage of specific indices (Zupo et al., 2017; Cpt. 6 of this thesis). Thus, the shape of biodiversity has been evaluated in a range of ecosystems and the results have been compared with the trends expected according to the above mentioned exponential decay relationship in order to validate the model here proposed, using both field data and mathematical forecasts.
Chapter 3.2. Food numerical abundance and pathways in seagrass food webs

Summary

3.2.1 **A study on the food web of *Posidonia oceanica* ecosystem: analysis of the gut contents of echinoderms.** *(cited by 38. IF=n.d.)*  
The analysis of the gut contents of 23 species of echinoderms indicates that this taxon is fundamental for the circulation of energy produced by *Posidonia oceanica* leaves. However this analysis is uniquely based on numerical abundance of prey.

3.2.2. **A study on the food webs of *Posidonia oceanica* (L.) Delile ecosystem: analysis of the gut contents of decapod crustaceans.** *(cited by 19. IF= n.d.)*  
Here the food webs of a key taxon for the ecology of *Posidonia oceanica* is studied based on the numerical abundance of prey in the gut contents. The study indicates that decapod crustaceans are generally to be considered as opportunistic detritivores and they play a key role in the transfer of energy to higher trophic levels.

3.2.3. **Depth and seasonal distribution of some groups of the vagile fauna of *Posidonia oceanica* leaf stratum: structural and trophic analyses.** *(cited by 193. IF= 1.79)*  
The crustacean decapods living in a protected area are important links between primary producers and final consumers in local food webs and their gut contents were analysed evaluating their numerical abundance. The results have been correlated to the functional algal groups in order to detect their influence in the structure of decapod communities. Therefore, both the influence of algae on the taxon and the influence of decapods on their environment were considered by taking into account the numerical abundance of prey in gut contents.
INTERNATIONAL WORKSHOP ON POSIDONIA OCEANICA BEDS

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A STUDY ON THE FOOD WEB OF THE POSIDONIA OCEANICA ECOSYSTEM:
ANALYSIS OF THE GUT CONTENTS OF ECHINODERMS

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A STUDY ON THE FOOD WEB OF THE POSIDONIA OCEANICA ECOSYSTEM: ANALYSIS OF THE GUT CONTENTS OF ECHINODERMS

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RIASSUNTO

Nell'ambito di una ricerca volta a definire le reti trofiche delle fauna vagile vivente nelle praterie di Posidonia oceanica (L.) sono stati esaminati i contenuti stomacali degli Echinodermi. I campioni sono stati prelevati in 35 stazioni attorno all'Isola d'Ischia (Golfo di Napoli) per mezzo di una gangamella su praterie di Posidonia oceanica per un periodo di circa un anno.

Sono state identificate 23 specie di Echinodermi (1 Crinoidea, 2 Holothuroidea, 6 Ophiuroidea, 9 Asteroidea, 5 Echinoidea). Nei contenuti stomacali di queste specie sono stati reperiti 90 taxa, 59 dei quali sono stati identificati sino al livello specifico e 15 sino a quello genetico.

Le categorie alimentari più comuni sono risultate essere: frammenti di Posidonia oceanica viva e morta (nel 77 % delle specie esaminate), Diatomee (nel 55 %), Molluschi (nel 50 %), Macrofiti (nel 36 %), Foraminiferi (nel 32 %), Poriferi (nel 32 %), Briozoï (nel 23 %), Policheti (nel 22 %).

Le specie mostranti una dieta meno specializzata sono: Holothuria tubulosa, Ophiura texturata, Paracentrotus lividus, Ophiocryptis fragilis, Ophioderma longicaudum, Echinocardium mortensi.

Tuttavia i frammenti di Posidonia oceanica dominavano quantitativamente nei contenuti stomacali di numerosi individui di Paracentrotus lividus ed Ophiura texturata, oltre che in quelli di Psammechinus microtuberculatus e Sphaerechinus granularis.

SUMMARY

In the frame of a research work carried out in order to define the food web of the vagile fauna living on the Posidonia oceanica beds, the stomach contents of Echinoderms were analyzed.

Samples were taken at 39 stations around the Island of Ischia (Gulf of Naples), by "gangamella" (bottom trawl) on P. oceanica beds, over a period of one year (day and night).

23 species of Echinoderms were collected (1 Crinoidea, 2 Holothuroidea, 6 Ophiuroidea, 9 Asteroidea, 5 Echinoidea).

Ninety taxa were detected in the stomach contents of these species, 59 of which were identified to the species level and 15 to the genus one.

The most common food items were: fragments of living and dead Posidonia oceanica (in 77 % of the examined species) Diatoms (55 %), Molluscs (50 %), Macrophytes (36 %), Foraminiferids (32 %), Sponges (32 %), Bryozoans (23 %), and Polychaeta (22 %).

The species showing a less specialized diet are: Holothuria tubulosa, Ophiura texturata, Paracentrotus lividus, Ophiocryptis fragilis, Ophioderma longicaudum, Echinocardium mortensi.

Nevertheless fragments of P. oceanica dominated in the gut contents of numerous individuals of Paracentrotus lividus and Ophiura texturata, besides Psammechinus microtuberculatus and Sphaerechinus granularis.
Dans le cadre d'un travail de recherche visant à définir le comportement alimentaire de la faune vagile vivant dans les herbières à Posidonia oceanica, les contenues stomacaux des Echinoderms ont été analysés. Des échantillons ont été prélevés en 39 stations autour de l'île d'Ischia (Golfe de Naples) à l'aide d'un chalut à oursin ("gangi") dans les herbières à Posidonia oceanica, sur une période d'une année, de jour et de nuit. 23 espèces d'Echinodermes ont été récoltées : 1 Crinoïde, 2 Holothuries, 6 Ophiurides, 9 Astéroïdes et 5 Echinofides. 20 taxons ont été différenciés dans les contenus stomacaux de ces espèces, 59 d'entre eux déterminés jusqu'au niveau de l'espèce et 15 jusqu'au genre. Les nourriture les plus communes étaient, si l'on considère le % d'espèces où elles ont été trouvées : fragments de feuilles vivantes et mortes de Posidonia oceanica (77 %), Diatomées (55 %), Mollusques (50 %), Macrophytes (36 %), Foraminifères (32 %), Porifères (32 %), Bryozoaires (23 %) et Polychètes (22 %). Les espèces ayant le régime alimentaire le moins spécialisé sont : Holothuria tubulosa, Ophiura texturata, Paracentrotus lividus, Ophiotrix fragilis, Ophioderma longicaudum, Echinocardium mortenseni. Cependant, des fragments de P. oceanica dominent dans les contenus stomacaux de nombreux individus de Paracentrotus lividus, Ophiura texturata, Psammechinus microtuberculatus et Sphaerechinus granularis.

1 INTRODUCTION

Although there are several recent studies on the structure of the food web in Posidonia oceanica ecosystem (LORENZI & FRESI, 1983 ; CHESSA et al., 1983 ; TRAER, 1980 ; VERLAQUE & NEDELEC, 1982), there is still a remarkable lack of information on the ways through which energy is canalized and distributed. The focal point seems to be the conversion of Posidonia biomass.

This is supposed to occur mainly via detritus and debris consumers, while the role of either true or occasional herbivores, with a few exceptions, is thought to be negligible, at least under normal circumstances. Much attention has been paid, in this regard, to some Echinoderms, with special regard to Echinoids and Holothuroids (VERLAQUE et al., 1981 ; TRAER, 1980 ; VERLAQUE, 1981). Paracentrotus lividus, in particular, has been intensively studied, due to its abundance in the superficial prairies and its possible two-fold role of herbivore and detrivore.

Other Echinoderms, with the remarkable exception of the genus Holothuria, have been essentially overlooked, notwithstanding the fact they represent an important fraction of Posidonia-associated macrobenthos.

In the present paper, the feeding behaviour of the Echinoderms, both as individual taxa and on the whole, is considered, in the attempt to ascertain at least from a qualitative standpoint, the position which the syntaxon occupies in the prairie food web.

This is done by means of gut contents analysis which we consider to be the most straight-forward and informative method in the study of the food webs.

2 MATERIALS AND METHODS

Material was sampled by means of a beam trawl (CHESSA, 1980 ; SANTARELLI & MICALE, 1963), in several prairies around the Island of Ischia, at depths ranging from 5 to 33 meters (Fig. 1).

After collection, animals were deep frozen and successively preserved in 70 % alcohol. Species were identified, mainly on the basis of TORTONESE (1965), and their gut contents analyzed and identified at the finest possible taxonomic level.

Several individuals of each species were studied : their gut content (both qualitatively and quantitatively) was then cumulated in a species "diet spectrum".

This material was then categorized into food items, the abundance of which was
Fig. 1: Map of the sampled area. 
= summer sample;  = winter sample;  
= Posidonia limits.

It is worth noting that Ophiotrix quinquecujula and Ophiotrix fragilis are now considered as belonging to the same taxon (Tortone, 1977). In order to test this hypothesis through their trophic behaviour we preferred to keep the two taxa as separate species.

Gut contents were categorized in several food items, 15 of which (living Posidonia, dead Posidonia, Dynoflageltes, Diatoms, Radiolarias, Foraminiferids, Sponges, Bryozoans, Decapods, Crustaceans, Coccocliothoforids, Gastropods, Bivalves, Polychaeta and Macrophytes) were retained for the analysis. Dead Posidonia was the most frequent and abundant food item, followed by Diatoms, living Posidonia, Macrophytes and Sponges. Echinoids such as Paracentrotus lividus, Sphaerchinus granularis, Psammochinus microtuberculatus and Echinocardium mortenseni are characterized by high abundance of vegetal material (living Posidonia, dead Posidonia, Macrophytes and Diatoms) occurring in almost equal proportions. Holothuria tubulosa, Ophiura texturata

3 RESULTS

Twenty-three species of Echinoderms were identified in our samples, for a total of 784 individuals (Tab. 1). Rare species (6) were not included in the analysis of the gut content which therefore took place on 270 individuals belonging to 17 different species. Among these, Holothuria tubulosa, Psammechinus microtuberculatus, Antedon mediterranea, Paracentrotus lividus, Ophiotrix fragilis and Ophiotrix quinquecujula were the most frequent ones.

coded by $\varnothing$ to 4 arbitrary scores.

The matrix species-food items was analyzed by the RQ autovectorial technique, which allows the simultaneous representation of variable-and observation-points in the same factorial plane.

The significance of the results was tested by means of the method proposed by Frontier (1974).
and Ophioderma longicaudum show a similar diet spectrum although dead Posidonia is the most abundant item (and for H. tubulosa almost all items appear with important abundance). Luidia ciliaris, Ophiomorpha fragilis and Ophiomorpha quinquemaculata show a living Posidonia, dead Posidonia and Diatom-based diet.

An entirely different spectrum is shown by Antedon mediterranea, that seems to feed primarily on planktonic microflora and microfauna, and by the two species of Astropecten whose diet is composed, almost exclusively, by Gastropods and Bivalves.

The RQ analysis, performed on the connection matrix yields three significant eigenvalues, accounting for, respectively, 35.76%, 20.59% and 12.64% of the system variance, for a total of 68.99%.

The correlation between species and food items coordinates is 0.65 in F1 and 0.49 in F2 set.

The resulting ordination model is visible in fig. 2: three main cluster are recognisable:

- cluster A at the negative pole of F1, mainly defined by Bivalves and Gastropods; it includes Astropecten aranciacus and A. irregularis;

- cluster B occupies a baricentric position and includes the majority of species and food items; the most central portion of this cluster is defined by living Posidonia, dead Posidonia, Decapods, Macrophytes, Foraminifera,

Fig. 2: Ordination model. Numbers indicate the species (see tab. I).

DY=Dynoflagellata; RA=Radiolaria; CO=Coccolithophorida; BR=Bryozoa; BI=Bivalvia; GA=Gasteropoda; CR=Crustacea; SP=Pteropoda; DIA=Diatomeae; FO=Foraminifera; DE=Decapoda; MA=Macrophyta; PM=dead Posidonia; PV=living Posidonia; PO=Poliachaeta.
Diatoms, Sponges and Crustaceans. Its fourth quadrant-satellite includes Echinaster sepositus and Holothuria tubulosa associated with Bryozoans and Coccolithophorids; - cluster C solely contains Antedon mediterranea and is defined by Dynoflagellates and Radiolarians. The introduction of axis 3 does not alter this configuration except for the fact that the satellite sub-cluster Bryozoans and Polychaetes is more clearly isolated from the central one which remains very compact.

4 DISCUSSION AND CONCLUSIONS

The picture which is obtained on the basis of the above results seems to be rather clear (Fig. 3) and agrees with a number of observations already done by other authors. In fact, beside a "carnivore pole" represented by the Astropecten species (which are known to feed primarily on Bivalves and Gastropods) and the "microfagous suspension feeders" pole represented by Antedon, all the other Posidonia bed Echinoderms can be lumped into a broad category of opportunistic omnivores whose diet seems to be differentiated only by their feeding type (deposit feeders, both specialized and unspecialized, browsers, etc.). Species such as Holothuria tubulosa and Echinocardium cordatum on one hand, and Paracentrotus lividus, Pseudocentrotus microtuberculatus, Ophiomia pentagona and Ophiura texturata on the other hand, are good examples for this heterogeneity of food items possibly dependent on the feeding mode.

There is little doubt, however, that this heterogeneity is more apparent than real when we consider:

1- the overwhelming importance of macrophytes-derived material in most of the species;

Fig. 3: Trophic relationships of Echinoderms in Posidonia oceanica beds.

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2- the close association between living Posidonia, dead Posidonia, Macrophytes and, for example, Diatoms and Foraminiferids (such groups could well be epiphytic, although this is not yet ascertained) that almost never occur in disjunction and form the core of cluster B in the ordination model.

3- the unselective feeding mode of several species, due to which "spurious" food items occur in an essentially vegetal-based diet.

We may conclude that, with few clear exceptions, the Echinoderms associated to Posidonia beds utilize detritus and debris of variable particle size issued from primary producers.

In principle, this is not obligatory, for they seem capable of utilizing various energy sources, a fact that would explain their essentially unspecialized feeding modes.

Echinoids (Paracentrotus lividus, P. microtuberculatus and Sphaechinus granularis) are apparently more linked to dead vegetal material, but they seem capable of shifting to graze on both live Posidonia and its epiphytic macrophytes and microphytes, when necessary.

The relative importance of these two feeding modes, in natural conditions, is still to be observed and our results do not provide evidence for either of them. Echinoderms should be considered as important converters of both live and dead vegetal material. The relevance of their contribution to higher trophic levels, however, is still to be evaluated.

While it is likely they remarkably contribute to the pelagic secondary production (Larvae), it might be hypothesized that their role in the Posidonia ecosystem is less important than that of, for instance, the class of Crustacea, due to a substantially lower degree of predation.

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A study on the food web of the Posidonia oceanica (L.) Delile ecosystem: analysis of the gut contents of Decapod Crustaceans.

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Summary. In the framework of a research carried out in order to define the food web of the vagile fauna living on Posidonia oceanica beds, the gut contents of Decapod Crustaceans are analyzed. The analysis of data indicates that most Decapod species have "opportunistic-detritivorous" feeding habits.

Résumé. On examine la position de quelques espèces de Crustacés Décapodes dans le réseau trophique de l'herbier de Posidonies de l'Île d'Ischia. L'analyse des données montre que la majorité des espèces a un comportement alimentaire de type détritivore-opportuniste.

- INTRODUCTION-
The trophic complexity of a seagrass ecosystem is very high because of numerous intermediates of the food web (Nelson, 1981). As far as Posidonia oceanica ecosystem is concerned, it is claimed that energy transfer from primary producers to higher trophic levels occurs mainly via "detritus food chains" (Ott & Maurer, 1977).

Feeding behaviour of individual taxa can offer an insight in the structure of the food web, particularly when, as in the case of Decapod Crustaceans, they are frequent and occupy a wide range of trophic positions.

In the mainframe of an investigation carried out to define the structure of the food web of the vagile fauna living on Posidonia oceanica prairies around Ischia (Gulf of Napoli), the trophic position of Decapods as syntaxon was analyzed.

The study was performed by examining their gut contents.

- MATERIALS AND METHODS -

Samples were taken by means of a "Gangamella" (Cfr. Chessa, 1980; Santarelli & Micale, 1963) having a 4 cm. mesh, in Posidonia oceanica beds around the Island of Ischia, at depths ranging from 5 to 33 meters. The animals from each sample were deep frozen and then preserved in alcohol 70%. The identification of the species was carried out mainly on the basis of the Zariquei-Alvarez (1968) classification.
The gut contents of each individual was examined and identified to the lowest possible taxonomic level. The abundance of each food item was arbitrarily coded from 1 to 4. 12 food items (Tab. 1) were entered in a "species/food items" matrix which was analyzed by the autovectorial technique known as "A.F.C.". This technique was used because it allows the simultaneous representation of both variable and observation points in the same factorial space.

The significance of the axes was tested by the method proposed by Frontier (1974).

Tab. 1: Food items considered in the analysis-
1) Green Posidonia (gp) 5) Bryozoa (br) 9) Amphipods (an)
2) Brown Posidonia (bp) 6) Sponges (sp) 10) Echinoderms (ec)
3) Algae (ma) 7) Polychaetes (po) 11) Molluscs (no)
4) Foraminiferids (fo) 8) Copepods (co) 12) Diatoms (di)

Tab. 2: Species considered in the analysis:
1) Clibanarius erythrurus 6) Inachus dorsettensis 11) Munida intermedia
2) Dorippe lanata 7) Inachus thoracicus 12) Paguristes oculatus
3) Ethusa mascarone 8) Palemon xiphias 13) Pagurus prideauxi
4) Eurynome aspera 9) Macropodia rostrata 14) Parthenope massena
5) Inachus communissimus 10) Maya verrucosa 15) Pisa muscosa
16) Pisa nodipes
17) Processa macrophthalma

- RESULTS -

38 species of Decapods were collected and identified (Tab. 2) for a total of 425 individuals; only the most frequent 17 species were considered in the analysis. Among these, the most abundant were: Eurynome aspera, Pagurus prideauxi, Paguristes oculatus and Macropodia rostrata.

The most frequent food items were: brown Posidonia (20.7%), Algae (17.7%), Diatoms (11.8%), Sponges (7.69%), Polychaetes (7.69%), living Posidonia (6.5%), Bryozoa (5.9%).

Brown Posidonia and Algae seem to be the most abundant food items.

The A.F.C. analysis, performed on the "species/food items" matrix, yields 3 significant eigenvalues, accounting for, respectively, 27.5%, 23.5% and 15.5% of the system variance.

The resulting ordination model is shown in Fig. 1. It is possible to observe that the majority of Decapods are grouped in a large cluster which contains both plant and animal food items.

This cluster is elongated in the space of F2 so as two poles are recognized. The first one is characterized by food item-points "Sponges"
and "Algae"; the second consists of Posidonia and its epiphytes (Bryozoa, Diatoms). Pagurus prideauxi is ordinated in the 1st quadrant; food items Foraminifera and Molluscs appear to be linked to this species.

Lastly the food items Copepods and Amphipods are segregated in the positive space of F1, linked to the species Palemon xiphias.

Fig. 1: Ordination model performed by an A.F.C. technique.

--- DISCUSSION AND CONCLUSIONS ---

The ordination model clearly shows that the majority of the species and food items are centrally ordinated. Such a configuration does not allow to identify a sharp difference in trophic strategy of the analyzed species, also because the cluster remains compact even when F3 is added.

This seems to indicate that most Decapods have "opportunistic detritivorous" feeding habits, although some species tend to prefer algae and other species tend to feed on Posidonia oceanica and its epiphytes.

The only two species which seem to prefer a carnivorous diet are Pagurus prideauxi and Palemon xiphias, according with the results of Chessa et al. (1983).
References:
Depth and Seasonal Distribution of Some Groups of the Vagile Fauna of the *Posidonia oceanica* Leaf Stratum: Structural and Trophic Analyses

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With 9 figures

Key words: Seagrasses, *Posidonia oceanica*, vagile fauna, depth gradient, seasonality, feeding guilds.

Abstract. The taxonomical and trophic structures of the vagile fauna communities of the leaf stratum in a *Posidonia oceanica* meadow at Ischia (Gulf of Naples, Italy) were investigated at five stations along a depth gradient (1 to 25 m). Sampling was performed in July, November, February, and May. The analyzed groups—polychaetes, molluscs, tanaids, isopods, amphipods, and decapods—exhibited similar distributional trends in all seasons, with coenotic discontinuities occurring at well-defined depths. The same zonation pattern was produced by feeding-guild analysis. Eleven trophic groups were identified. The most abundant groups were: Herbivores, which were found mainly at the shallow stations; Herbivores-deposit feeders, which were widely distributed along the transect; Deposit feeders-carnivores, found mainly at the deep stations.

This study suggests that in the *Posidonia* leaf stratum, herbivores and herbivores-deposit feeders, as consumers of epiphytic micro- and macroflora and deposited particulate organic matter, play an important role in the energy transfer from producers to higher trophic levels of the system.

Problem

The vagile fauna within the *Posidonia oceanica* canopy constitutes one of the most important components of the ecosystem formed by this phanerogam (Kikuchi & Pères, 1977). A relevant fraction of these vagile organisms, both in term of abundance and species diversity, is composed of forms linked to the leaf surface (Ledoyer, 1968). They are strongly influenced by the features of both the plant, which is a growing substrate with high seasonality (Ött, 1980), and its epiphytes (Mazzella & Ott, 1984), showing specific adaptations to the various microenvironments.

Only in recent years have studies focused on the structure of some components of the vagile communities in relation to the environment (Scipione et al.,
Within this depth gradient, changes in parameters such as hydrodynamic forces, light, temperature, and sediment structure determine differences in meadow structure and, directly and indirectly, in the structure and distribution of the communities. This has also been observed at the level of individual taxocoenes such as polychaetes (Gambi et al., 1989), mollusces (Russo et al., 1984; 1991), isopods (Lorenti & Fresi, 1983), amphipods (Scipione & Fresi, 1984), and decapods (Zupo et al., 1989).

These studies revealed the presence of a well-defined community associated with the shallowest stand of the Posidonia bed, and of deeper communities with site-specific depth ranges.

The trophic interactions in the Posidonia ecosystem are highly complex (Ott, 1981; Chessa et al., 1983) and influence community structure. Studies on the functioning of this system have mostly stressed the role of the detritus-chain in energy transfer (Ott, 1981; Wittmann et al., 1981), placing less emphasis on the grazing activity on epiphytes. This aspect, however, is of paramount importance in trophic interactions at the leaf stratum level; it has been recently addressed in individual taxocoenes by means of feeding-guild and morpho-functional analyses (Russo, 1989; Scipione, 1989; Gambi et al., 1989b) as well as by an experimental approach (Mazzella & Russo, 1989; Scipione & Mazzella, in press). Such investigations suggested the presence of links and interactions between plant epiphytes and animals, pointing out also interesting implications regarding co-evolution (Russo, 1986; Mazzella et al., in press).

The present analysis takes into account community structure and its trophic composition. It is based on feeding guilds and considers various groups such as polychaetes, mollusces, tanaids, isopods, amphipods, and decapods, which previous studies have revealed to be relevant components – in terms of abundance and species richness – of the vagile fauna of the leaf stratum. This study aims at determining how the depth distributional pattern is maintained over time (seasons), both in taxon and trophic guild organization. This should provide basic knowledge of how biotic interactions (e.g., feeding behaviour) contribute, together with abiotic factors, to determine the structure of the communities.

**Material and Methods**

Investigations were carried out in a Posidonia oceanica bed off Lacco Ameno (Island of Ischia, Gulf of Naples), which extends from 1 m to about 33 m depth (Colontoni et al., 1982; Mazzella et al., 1989). Five stations, located along a depth transect at 1 m, 3 m, 10 m, 15 m, and 25 m, were studied. At each station, two replicate samples of vagile fauna were collected in July and November (1981) as well as in February and May (1982), for a total of 10 samples a month. Samples were collected using a hand-towed net (a rectangular frame measuring 40 x 20 cm with a net of 400 μm mesh size) by SCUBA diving according to the technique described by Ledoyer (1962) and modified and standardized by Russo et al. (1985) and Russo & Vinci (1991). This technique consists of a series of strokes (60) delivered in such a way as to shake the Posidonia leaves from the base of the shoots (Russo et al., 1985). The method is semi-quantitative and samples can therefore be compared. However, the variety of microenvironments in the Posidonia canopy and the different sizes and behaviours or life habits of organisms result in different capture efficiencies for the various groups. Nevertheless, in our opinion this method is best suited to collect those vagile organisms strictly associated with the
with terms (17.6 51.4 ... individuals. Taxa by a mean linkage cluster (2.1 ... observations). According to literature data. Due to the different trophic behaviours often exhibited by the same species, mixed categories were created. Species with unknown trophic behaviour were assigned to the category Unknown (Un). A total of 11 feeding guilds were identified: Suspension feeders (SF), which feed on seston; Deposit feeders (DF), which feed on surface detritus; Deposit-suspension feeders (DSF), which feed both on deposited and suspended material; Deposit feeders-carnivores (DC), which feed on deposited material and fauna; Liminvores (Li), which feed on buried organic material; Herbivores (He), which graze on micro- and macroalgae; Detritus feeders (DeF), which feed on Posidonia tissue as "detritus"; Herbivores-deposit feeders (HeDF), which feed on plant epiphytes and trapped organic material; Carnivores (Ca), which include predators or scavengers; Omnivores (Om), which may behave as carnivores, herbivores, and/or deposit-feeders; Parasites (Pa), which parasitize other animals.

Population parameters such as species richness, abundance, Qualitative Dominance (DQ % = % number of species belonging to a given taxon), Quantitative Dominance (DI % = % number of individuals belonging to a given taxon), and trophic Quantitative Dominance (% number of individuals belonging to a given feeding guild) were calculated (BOUJOURSQUE, 1971). Structural analysis of the community was performed through a Correspondence Analysis (CA) (LEGENDRE & LEGENDRE, 1984) on a reduced set of species (variables), eliminating those occurring only with one individual in all the samples (40 observations). Significance of the CA axes was calculated according to FRONTIER (1974). Differences between the two replicates of each station were compared by the Wilcoxon test (SOKAL & ROHLE, 1973). In order to evaluate intra- and intersample similarity, a mean linkage cluster analysis was performed on the centered scalar product matrix obtained by the "species vs. stations" analysis.

A functional analysis was performed using again the Correspondence Analysis on the 11 identified trophic categories (variables) and pooling the two replicates of each station (20 observations).

Results

1. Descriptive taxonomic and trophic analysis

The examined taxonomic groups were represented by 312 species and 20,591 individuals. Taxa with the highest number of species included Mollusca (DQ: 30.0 %), Polychaeta (27.2 %), and Amphipoda (26.6 %), while Decapoda (7.6 %), Isopoda (6.2 %), and Tanaidacea (2.4 %) were less represented. In terms of numbers of individuals, the best represented taxa were Mollusca (DI: 51.4 %), Decapoda (23.1 %), and Amphipoda (21.0 %), followed by Polychaeta (2.1 %), Isopoda (1.8 %), and Tanaidacea (0.6 %).

Community abundance was highest in November (7,622 individuals for 10 samples), intermediate in February (4,919), and low in July (3,772) and May (3,772). Molluscs dominated in all seasons, particularly in February (58.2 %), with the exception of May (38.1 %), when amphipods (33.9 %) were also dominant. This latter taxon was constantly abundant, particularly in July (22.7 %). Decapods were important in November (28.2 %) and February (17.6 %) (Fig. 1a).
Fig. 1. Quantitative (DQ %) (a) and qualitative (DQ %) (b) dominances of vagile fauna of the Posidonia oceanica leaf stratum in different seasons. a: July (J): 1.2% (Po), 52.3% (Mo), 10% (Ta), 2.2% (Is), 22.7% (Am), 20.6% (De). November (N): 0.8% (Po), 53.2% (Mo), 0.3% (Ta), 0.8% (Is), 16.7% (Am), 28.2% (De). February (F): 4.0% (Po), 58.2% (Mo), 0.8% (Ta), 0.8% (Is), 16.7% (Am), 20.6% (De). May (M): 3.2% (Po), 38.1% (Mo), 0.4% (Ta), 2.0% (Is), 33.9% (Am), 22.4% (De). b: July (J): 13.5% (Po), 38.7% (Mo), 0.8% (Ta), 8.4% (Is), 27.7% (Am), 10.9% (De). November (N): 21.5% (Po), 38.0% (Mo), 1.8% (Ta), 6.8% (Is), 25.8% (Am), 6.1% (De). February (F): 24.4% (Po), 27.3% (Mo), 2.5% (Ta), 6.3% (Is), 30.7% (Am), 8.8% (De). May (M): 18.4% (Po), 34.4% (Mo), 1.8% (Ta), 7.4% (Is), 31.3% (Am), 6.7% (De).

The taxa with the highest number of species in all seasons were molluscs, particularly in July and November (DQ: 38.7% and 38.0%, respectively), followed by amphipods, particularly in February and May (30.7% and 31.3%, respectively), and polychaetes, mainly in February (Fig. 1b).

The number of species along the transect increased slightly with depth, particularly in February, when the community had the lowest values at 1 m (mean of the two samples: 27) and the highest at 25 m (87). The lowest overall values were observed in July, ranging from 34.5 species at 1 m to 44.0 at 25 m. At 1 m the highest values were observed in May (64). At 3 m and 10 m in February and November, the number of species showed complementary trends (Fig. 2a).

The number of individuals was highest at 3 m (3,462.5 total value, mean of the two replicates) in all the considered months, with an exceptionally high value in November (1,816; mean of the two replicates). In May, however, the highest values were found at 1 m (520.5), where the lowest values occurred in February (151.5) (Fig. 2b).

The trophic analysis revealed that the dominant feeding categories of the community as a whole were Herbivores-deposit feeders (60.7%), Deposit feeders-carnivores (16.0%), Herbivores (10.5%), and Carnivores (4.9%).
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Fig. 2. Number of species (a) and number of individuals (b) of vagile fauna in the four seasons along the depth transect. The columns represent the mean values of the two replicates, the bars the single values of each replicate.
Fig. 3. Number of individuals belonging to the different feeding guilds along the depth transect and in the different seasons. The number of individuals for He, HeDF, and DC have been divided by 10 in order to maintain the same numerical scale on the Y axis.
followed by Deposit-suspension feeders (2.4 %), Deposit feeders (1.9 %), Unknown (1.5 %), Omnivores (1.4 %), Parasites (0.3 %), Detritus feeders (0.2 %), and Suspension feeders (0.2 %).

The HeDF dominated along the transect in all seasons, reaching their highest values mainly at 3 m. Herbivores were most abundant at the shallow stations in May (599 indiv. at 1 m; 244 indiv. at 3 m). Ca and DC were most common at intermediate and deep stations, the latter group attaining highest values in November at 3 m and 10 m (803 and 367 indiv., respectively). Other groups showed no particular distributional trends, exhibiting maximum number of individuals in different seasons and depths (Fig. 3).

**Polychaeta**

A total of 934 individuals belonging to 101 taxa were collected; 40 species were represented by only a single individual. A total of 506 individuals, belonging to 22 species of the families Terebellidae, Sabellidae, Serpulidae, and Spirobranchidae, were sessile forms living permanently within tubes. Due to their life habits, these suspension-feeders are omitted from the following analysis, which therefore refers to the 79 remaining vagile or sedentary species.

Among the many species found, none was notably more abundant than or dominant over the others. The best represented family was Syllidae, which had the highest number of species (40) yet relatively few individuals (270). The total number of species and individuals increased from July to February, when maxima were observed, and decreased slightly in May. In each month, abundance generally increased with depth, while the number of species showed irregular trends.

With regard to spatial distribution, some species were present or slightly more abundant in the shallowest stands (1 and 3 m): Grubeosyllis clavata (Claparede), G. vieitezii San Martin, Syllis prolifer KROHN, Platynereis dumerilii (Aud. & M. Edw.), and Polyophthalmus pictus (Dujardin). Other species occurred or were more abundant in the intermediate and deep stands: Kefersteinia cirrata (Keferstein), Grubeosyllis yraide San Martin, Eurysyllis tuberculata Ehlers, and Odontosyllis gibba Claparede. The remaining taxa were present at all depths (Sphaerosyllis hystrix Claparede) or showed an irregular distribution pattern.

The few species present during all months were S. hystrix, E. tuberculata, P. dumerilii, and Glycera tesselata Grube. Seasonal differences were due to a few species being more abundant in a particular month, e. g. G. clavata and S. prolifer in May, K. cirrata and S. hystrix in November, or E. tuberculata and P. pictus in February. The remaining taxa showed no particular temporal pattern.

The trophic analysis yielded a total of nine feeding guilds, most coherent with the classification of FAUCHALD & JUMARS (1979). Carnivores (181 individuals in all samples), mostly K. cirrata and several species of the genus Syllis (Syllidae), were the most abundant guild and generally inhabited the deeper stations. Herbivores (77 individuals), mainly P. dumerilii, Nereis rava Ehlers, and P. pictus, were quite abundant, especially in the shallow stations and in July and
May. Deposit feeders-carnivores (53 individuals) were represented by species of the genus *Sphaerosyllis* (*Syllidae: Exogoninae*) and were relatively abundant along the entire transect in November and February, being negligible in the other months. Herbivores-deposit feeders (82 individuals), composed of different *Exogoninae*, were present all along the transect and were more abundant in May. The few Deposit feeders and Limivores most likely included species inhabiting the rhizomes.

**Mollusca**

A total of 10,330 individuals belonging to 87 species – 20 being represented by only one specimen – were identified. They were distributed in two classes as follows: *Gastropoda* (no. of indiv.: 10,292; DI: 99.7%; no. of species: 76; DQ: 87.4%), *Pelecypoda* (32; 0.3%; 11; 12.6%).

The prosobranch gastropod groups with the highest number of individuals and species were *Trochidae*, in shallow beds, *Cerithiidae*, in deep beds, and especially *Rissoidae*, at all depths (see also Russo et al., 1983). As stressed earlier (Idato et al., 1983), the structure of the communities along the depth gradient showed a clear zonation pattern. Such structural zonation, with changes in population parameters (number of species and individuals), was also found to persist seasonally (Russo et al., 1984; 1991).

Six feeding guilds were identified, mainly on the basis of radular structure and, therefore, of the main evolutionary trends as reflected by prosobranch systematics (Russo, 1986). The *Archaeogastropoda* group contains most truly grazing Herbivores inhabiting seagrass beds (e.g., *Gibbula* spp., *Jujubinus* spp.); it also includes species which feed on colonial and sedentary animals (FCSA, e.g., *Calliostoma* spp.) as defined by Purchon (1977), a category distinguished from true Carnivores. This latter group also contains some mesogastropods (*Cerithiopsis* spp. and *Momorphus* spp.). Most *Mesogastropoda* in the *Posidonia* beds (mainly *Rissoidae* and *Cerithiidae*) have been grouped in the Herbivores-deposit feeders guild. The most evolved group, *Neogastropoda*, mainly specialised as active predators, has been included in the Carnivores guild.

The category Suspension feeders was composed solely of the few sestonophagous bivalves collected by the hand-towed net. Herbivores-deposit feeders were the most abundant all along the transect and in all seasons (Fig. 4); species that contribute to the high quantitative dominance (84.9%) of this guild were *Rissoa* (*Goniostoma* *italiensis* Verduin, in the shallow bed (1–3 m), *Alvania lineata* Risso, at intermediate depths (3–10 m), and *Rissoa violacea* Desmarest and *Bittium latreilli* (Payraudeau) in the deep bed (15–25 m). Herbivores mainly inhabited the shallowest station (1 m) and showed a peak abundance in February, due mainly to *Gibbula ardens* (von Salis) (Fig. 4). Carnivores increased in dominance with depth, reaching maximum values in the deepest stations in all seasons, mainly due to *Granulina clandestina* (Broccoli). A minor role was played by the other feeding guilds such as Parasites, FCSA, and Suspension feeders, which seemed to be more prevalent in the deep stands.
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Fig. 4. Mollusca. Quantitative Dominance (DI %) of trophic categories along the depth transect in the four seasons.

Tanaidacea

Six species of tanaids were found, for a total of 103 individuals. Of these, 67 individuals were Leptochelia savignyi KROYER, which is present in all four seasons, reaching a maximum number in July (39) and showing a decreasing trend with depth. Parapseudes latifrons (GRUBE) and Paratanidae gen. sp. were found only in February in the shallow and deep stands of the bed, respectively. The other species were present with few individuals in only one station.

The collected species were considered to be Deposit feeders; some forms (Apseudidae) can supplement deposit feeding behaviour with suspension feeding.

Isopoda

Isopods were present with 18 species and a total of 369 individuals. As a whole, the number of individuals reached a peak in February (148) compared with July (82), November (61), and May (77). The number of species did not vary significantly with season: July (10), November (11), February (13), May (12).

As regards depth distribution, the maximum number of individuals was found at intermediate stations (10, 15 m), except in February (25 m), while the minimum always occurred at 1 m.
Trends of the whole taxon were strongly influenced by *Cymodoce hansenii* Dumay (179 individuals, mostly juveniles), which was by far the dominant species in November (DI: 55.7 %), February (55.0 %), and May (63.6 %); in July this role was played by *Synisoma appendiculatum* (Risso) (40.0 %). Furthermore, *Disconectes picardi* (Amar) reached its peak of abundance in July, whereas *Jaeropsis dolffusi* Norman and *Dynamene tubicauda* Holdich were more abundant in February. Other species (*Gnathia cf. vorax* Lucas, *Astacilla mediterranea* Koehler) were present in all four months, although their distribution did not show any clear trend.

With regard to feeding guild distribution, Herbivores-deposit feeders were strongly dominant both in terms of species (DQ: 53.0 %) and individuals (DI: 70.0 %). Due to the numerical importance of *C. hansenii* and *S. appendiculatum* within this trophic group, temporal and spatial patterns of Herbivores-deposit feeders fundamentally reflected those shown by the two species. Parasites were represented mainly by juvenile *Gnathia*, known to feed on seagrass fishes. *J. dolffusi* was included among Carnivores, based on an observation by Fresi (1968) that this species may feed preferentially on *Hydrozoa*. Deposit-suspension feeders were represented by *A. mediterranea*.

**Amphipoda**

Amphipods were present with 77 species, belonging to 51 genera and 25 families, for a total of 4,217 individuals. Of these, 19 were rare (only one individual).

The number of species along the transect showed different trends in the different seasons: a slight increase with depth was recorded in July (mean of the two replicates from 9.5 to 13), while in February this increase was more pronounced. Here, minimum values occurred at 1 m (5), maximum values at 25 m (31), with a strong reduction at 10 m (11). A similar pattern of reduction was noted in May, when the highest values were present instead at 1 m (19.5). A trend opposite to that recorded in February and May was found in November, with the lowest values at 25 m (9).

The number of individuals increased with depth in February, with the highest values at 25 m (mean individuals 139) and the lowest at 1 m (11); an opposite trend was observed in the other months, particularly in May, when the greatest number was found at 1 m (343.5).

The amphipod community was mainly represented by *Apherusa chiereghinii* Giordani-Soika (total number of individuals: 903), *Amphithoe helleri* G. Karman (643), *Dexamine spinosa* Montagu (337), *Hyale schmidti* (Heller) (285), *Aora spinicornis* Afonso (273), *Phitica marina* Slabber (222), and *Liljeborgia dellavallei* Stebbing (100); these species accounted for 65.5 % of the total. In July, the dominant species were *D. spinosa* and *P. marina*, in November and February *A. spinicornis* and *A. chiereghinii*, while in May *A. helleri* was dominant, being responsible for the highest abundance values at 1 m, as noted above.

Eight trophic categories were identified: Deposit feeders (6.1 %), Deposit-suspension feeders (10.5 %), Deposit feeders-carnivores (2.9 %), Herbivores (30.2 %), Detritus feeders (0.8 %), Herbivores-deposit feeders (35.8 %),
Omnivores (6.7%), Unknown (7.0%). The dominant feeding categories, He and HeDF, were mainly represented by *H. schmidti*, *Hyale campionyx* (Heller), *A. helleri*, *Amphitoe ramondi* Audouin, *Cymadusa crassicornis* (A. Costa), as well as by *A. chiereghinii* and *D. spinosa*, respectively.

The highest Herbivore dominances were observed at the shallow stations, mainly in July (1 m: 57.8%; 3 m: 49.3%) and May (1 m: 75.2%; 3 m: 53.9%). Herbivores-deposit feeders were present constantly all along the transect, particularly in November (50.6%) and February (36.5%) (Fig. 5); in these seasons, Deposit-suspension feeders were also important (17.8% and 14.5%, respectively). In July the Omnivores, mainly represented by the *Caprellidae P. marina*, were also dominant (17.7%). In February and May, in the deeper stands, Deposit feeders such as *L. dellavallei* and Deposit feeders-carnivores such as *Lysianassidae* were also important. Detritus feeders were poorly represented by *Atylus vedlomensis* (Bate & West.) and *Gammarella fucicola* (Leach) and were present only in the deepest station (25 m).

**Decapoda**

Sixteen species, totalling 4,632 individuals, were identified; some, such as *Pisa muscosa* (L.) and *Macropodia rostrata* (L.), belonging to the family *Majidae*, were represented only by few individuals. The strongly dominant species in all
the considered months were *Hippolyte inermis* LEACH (1,419 indiv.) among the *Natantia* and *Cestopagurus timidus* (ROUX) (2,717) among the *Reptantia*. The remaining species were apparently related to particular seasons: *Galathea intermedia* LILLIEBORG more frequent in winter months; *Galathea bolivari* ZARIQUIEY ALVAREZ present only in July and February; few *Eury nome aspera* (PENNANT) in November; and *Thoralus cranchii* (LEACH) present only in November and February.

The two dominant species showed different patterns along the depth transect in the different months (Fig. 6). In July and November, *H. inermis* was more abundant at the shallower stations, in February at the intermediate stations (10–15 m). In May this species showed a strong decrease at 15 and 25 m. Compared with the distribution of *H. inermis*, *C. timidus* showed an opposite trend in July, a similar trend in November, while in February and May it was most abundant in the deeper stations.

Five trophic groups were identified. Due to the high abundances of *H. inermis* and *C. timidus*, their feeding behaviour strongly determined the trophic structure of the taxon. The former can be considered to be a Herbivore-deposit feeder, the latter a Deposit feeder-carnivore. In July, Deposit feeders-carnivores were more abundant at the deeper stations and Herbivores-deposit feeders at the shallow stations; in November they were equally abundant all.
along the transect, with a small peak of the former at 3 m (800 indiv.). The abundance of DC increased towards the deeper stations in February and May, when the HeDF were found mainly at the shallower stations. In February and May, Carnivores were also present in greater numbers.

2. Structural analysis

a. Population structure

The Correspondence Analysis was performed on 212 species: 45 polychaetes, 67 molluscs, 7 tanaids, 13 isopods, 63 amphipods, and 17 decapods. Two axes were significant and accounted for 19.2% and 15.0% of total variance, respectively. Observation points of all the months considered are ordered along the first axis according to depth, with the shallow station samples at the positive pole and the deep ones at the negative pole (Fig. 7). The second axis separates the majority of the samples according to the month of collection, especially the shallow and intermediate stations (1 m, 3 m, 15 m), with February and November toward the positive pole and July and May toward the negative pole; the shallow station samples (1 m, 3 m) of May are strongly separated from the others. The two replicate-points closely adjoin each other, except in certain shallow stations (e.g., 1 m in May); the distances between the replicate-points decreased with depth. The two replicates for each station were not significantly different at P = 0.05 (WILCOXON test). The cluster analysis also revealed that the replicates of most stations were associated at high levels of similarity; the exceptions were the stations at 1 and 10 m in July, at 25 m in November, and at 15 m in February, which were associated at an average similarity below 0.5.

The species contributing the most to axes I and II were located near the following stations:

- 1 m (May): *Grubeosyllis clavata* (II: 1.5), *Syllis prolifera* (II: 1.1), *Amphithoe helleri* (I: 21.8; II: 31.0), *Cymadusa crassicornis* (I: 1.3; II: 1.5), *Microdeutopus anomalus* (RATHKE) (I: 2.0; II: 4.1), *Elasmopus* sp. (II: 1.3), *Stenothoe monoculoides* (MONTAGU) (I: 1.1; II: 1.1), *Hyale camptonyx* (II: 1.3);
- 1 m (July): *Gibbula umbilicaris* (L.) (I: 2.4), *Hyale schmidti* (I: 3.1);
- 3 m (May–July): *Dexamine spinosa* (I: 1.1);
- 1 m (February): *Gibbula ardens* (I: 1.9; II: 1.7), *Rissoa italiensis* (I: 7.3; II: 14.8);
- 3 m (February): *Rissoa ventricosa* (DESMAREST) (II: 1.4); *Hippolyte inermis* (I: 1.5; II: 2.7);
- 3 m (November): *Alvania lineata* (II: 11.8);
Fig. 7. Population structure of vagile fauna. Ordination model of Correspondence Analysis (CA). 212 variables (species), 40 observations (samples). Station points: 1-3-10-15-25 (depths), a-b (July), c-d (November), e-f (February), g-h (May). Species points: 8, Grubeosyllis clavata; 28, Syllis prolifera; 49, Gibbula ardens; 50, Gibbula umbilicaris; 60, Rissoella inflata; 61, Coriandria cossurae; 64, Pusillina dolium; 68, Rissoa italiensis; 71, Rissoa ventricosa; 72, Rissoa violacea; 73, Alvania lineata; 77, Bittium laireillii; 102, Odostioma sp.; 130, Cymodoce hanseni; 143, Amphithoe hellerti; 145, Cymadusa crassicornis; 148, Microdeutopus anomalous; 152, Apherusa vexatrix; 158, Dexamine spinosa; 165, Elasmopus sp.; 187, Stenothea monoculoides; 188, Hyale camptonix; 189, Hyale schmidtii; 197, Hippolyte inermis; 204, Cestopagurus timidus.
Fig. 8. Trophic structure of vagile fauna. Ordination model of Correspondence Analysis (CA).
12 variables (trophic categories), 20 observations (samples). Station points: 1-3-10-15-25 (depths),
b. Trophic structure

The ordination model – in the plane of the first two factors, which account for the following percentages of variance, FI: 57.9% and FII: 18.9% – shows that the station points are ordered according to depth, along a series of parabolic arches, with the shallow and the deep stations toward the positive pole of axis I and II, respectively (Fig. 8). A strong disjunction of Herbivores, located close to the 1 m stations in May and February, is evident; the other main feeding guilds are ordered according to depth: Herbivores-deposit feeders close to the intermediate stations, Deposit feeders-carnivores and Carnivores toward the deepest stations along with Suspension- and Deposit feeders; Detritus feeders are strongly polarized toward the positive pole of axis II.

Discussion

The vagile fauna of the *Posidonia oceanica* leaf stratum is clearly zoned along the transect, showing almost the same pattern in the four seasons; this confirms the results of a two-month analysis carried out in the same meadow (Mazzella et al., 1989; Mazzella et al., 1991).

The zonation along the depth gradient reveals the presence of a shallower community, characterized by a more pronounced seasonality, and a deeper, less variable one. A transition can be recognized between these two assemblages; it corresponds to the intermediate portion of the meadow and is characterized by special features (Pirce, 1984) such as meadow morphology and clines of physical factors (e.g., thermocline, water motion). This zone is therefore a boundary between the shallow stand, where the environment is more stressful, and the more stable deep one.

In autumn and winter the communities were richer both in species and individuals, as has been observed for other phanerogams (Nelson, 1979; Nelson et al., 1982) and in previous analyses (Mazzella et al., 1989), and present a higher variability along the transect than in other seasons. This could be due to a number of seasonal factors, both abiotic, such as higher fluctuations of environmental conditions, and biotic. The latter include meadow features (reduced leaf covering and canopy height), lower predation pressure (Nelson, 1979), and individual taxon dynamics.

As for community trophic structure along the transect in the different months, a strong correlation with population structure was noted. This is evident from a comparison of the two CA ordinations (Figs. 7 and 8). Trophic structure is dominated by plant feeders (HeDF and He), which account for over 71% of total population and show a well-defined vertical zonation. This dominance is also evident within each faunistic group, for example in the dominant taxa, molluscs and amphipods. Individual taxonomic groups showed specific trends that will be discussed separately.

Polychaetes in the *Posidonia* leaf stratum had a high species richness but low abundances. This is coherent with previous studies in Lacco Ameno (Colognola et al., 1984; Mazzella et al., 1989) and in other prairies (Gambi et al., 1989b). Vagile polychaetes apparently do not find the leaf stratum a favorable
habitat, probably due to the more stressful physical conditions, competition with other taxa, or predation pressure (Chessa et al., 1989). Polychaetes are more abundant in the rhizome layer (Pronzato & Belloni, 1981; San Martin & Vieitez, 1984; Giangrande, 1985) where they generally find more favorable physical conditions (Gambi et al., 1989a). Among the dominant family of Syllidae, many species are interstitial forms (Exogoninae), while those syllids abundant in the shallow stand of the bed are commonly found on hard littoral bottoms covered by algae (Giangrande, 1988). This is the case in Platynereis dumerilii, one of the most abundant species in Posidonia beds and frequently also recorded in environments with low competition, such as polluted hard bottoms (Bellan, 1980). The life cycle of P. dumerilii has many traits of an r-strategy species (Giangrande, 1989); moreover, this species is a herbivore whose role in the decay of living and dead brown algae tissue on the Atlantic coasts has been well documented (Bedford & Moore, 1985). Its distribution in the shallowest Lacco Ameno stands coincides with the higher epiphytes development here (Mazzella et al., 1989) and suggests that the species is tropically linked to the plant epiphyte and/or leaf-detritus.

Gastropods almost exclusively characterize the mollusc community on Posidonia leaves. Some species preferentially inhabit this environment and have evolved K-strategies of reproduction (Russo, 1989). The high dominance of Herbivores in February at 1 m is due to the population dynamics of the dominant species (Gibbula ardens) and to its trophic specialization, well-adapted to the first colonization stages of the seagrass epiphytic covering (Mazzella & Russo, 1989). In deep stands, the presence of Pa, FCSA, and SF is coherent with the decreased shoot density and light intensity occurring in seagrass stands with higher sedimentation rates. This on one hand permits better growth of encrusting animals and higher abundances of their predator molluscs (FCSA, e.g., Monophorus spp.), and, on the other hand, important intrusions of soft bottom faunas such as bivalves (Suspension feeders) and echinoderms with their parasitic molluscs (e.g., Vitreolina devians). In general, only minor variations in feeding structure seem to occur during the year. This may be explained by the high abundance of Mesogastropoda at all depths and in all seasons; they are mainly grouped in a single guild, HeDF, which includes several trophic specializations. Future investigations on Mesogastropoda biology using a variety of methodological tools should enable us to split them into new, more precisely defined guilds. At present, more accurate information on their feeding strategies is lacking, especially for most Rissoidae and Cerithiidae.

The isopod taxon shows a temporal pattern, with peak abundance in February and no obvious variation in species richness between seasons, and a spatial pattern, with low abundance at shallower depths. At least five species can be considered part of the fundamental stock of the Posidonia community (Ledoyer, 1966; Lorenti & Freni, 1983). Only Cymodoce hansenii is strongly dominant, except in July, showing a depth-related distribution; other species show no clear trend, in part because of their low overall abundance. However, a comparison between the seasonal distribution of the two species which numerically dominate HeDF shows differences that may be due to their microhabitat range: S. appendiculatum, which is morphologically adapted to the leaf blade-epiphytes system, mostly occurs in May and July, when plant growth is at a
peak, while *C. hansenii*, which undergoes microhabitat shifts also on a diel basis (Lorenti & Scipione, 1990), shows a more even temporal distribution.

The amphipod population is one of the most important components of the *Posidonia* vagile fauna (Ledoyer, 1962; Mazzella et al., 1989). As food for decapods and fishes, it plays a major role in the energy transfer to higher trophic levels (Bell & Harmelin-Vivien, 1983; Chessa et al., 1983; Sparla, 1989) and is clearly zoned with depth in the Lacco Ameno meadow. A shallow water amphipod community, able to withstand high water movement and mainly represented by Herbivores in May and July, is related to the erect algal epiphytic layer; this layer is more developed in these months and constitutes a primary food source for amphipods. A community mainly represented by Herbivores-deposit feeders occurs at intermediate and deep stations, where major deposition of particulate organic matter on leaves may favour the presence of "detritus cleaner" forms; these forms represent the main trophic group in the foliar stratum, as has been observed in other phanerogams (Nagle, 1968; Howard, 1982; Lewis & Hollingworth, 1982) and in a previous analysis (Scipione, 1989). Deposit-suspension feeders and Deposit feeders-carnivores are better represented at the deep stations, probably because of the more heterogeneous meadow structure and an overlapping with surrounding soft bottoms. The different seasonal distributions of dominant species that are within the same trophic group - such as *Apherusa chireghinii* and *Dexamine spinosa* - are most likely due to different life cycles, competition phenomena, or various grazing adaptations to plant epiphytes (Greze, 1968). The detritus pathway, which constitutes an important means of energy transfer for amphipods (Wittmann et al., 1981) in *Posidonia* systems, appears to be negligible at the leaf stratum level for the presence of a separate amphipod community.

Previous studies have revealed a higher species richness in the decapod populations of *Posidonia* beds (Monchampont, 1979–80; Garcia-Raso, 1990; Zupo, 1990). The species identified here are typical of the leaf stratum as typically sampled by the hand-towed net method. The wide distribution and high abundances of *Hippolyte inermis* and *Cestopagurus timidus* along the transect is due to their adaptability to different hydrodynamic and trophic conditions; their life cycles are also well adapted to the plant's annual cycle (Pessani, in press; Zupo, submitted). Less abundant species seem to be linked to a narrower depth range. For example, *Clibanarius erythropolis*, as stated in a previous paper (Zupo et al., 1989), prefers shallow waters, while *Anapagurus laevis* is typical of deep waters. Herbivores-deposit feeders feed preferentially on diatoms and algal detritus, while Deposit feeders-carnivores - mainly limited to the deeper stations - feed on grazers such as copepods and small molluscs. Decapods, therefore, participate in both epiphyte and detritus food webs (Zupo & Fresi, 1985); as a food source for large predators (*e.g.*, fishes) (Bell & Harmelin-Vivien, 1983), they also represent an important path in the transfer of energy to the highest trophic levels.

As a whole, the vagile fauna distribution basically reflects the environmental gradient, although biological accommodation of single species - in terms of trophic relationships and life histories - plays a crucial role. In particular, the high dominance of Herbivores at the shallower stations in February and May is
consistent with patterns of algal epiphyte biomass (Fig. 9; MAZZELLA & BUJA, 1989). In summer, when epiphyte biomass is more evenly distributed along the transect, herbivore presence is more uniform (see the minor polarization of July station points in the ordination model in Fig. 8). It should be noted that herbivore dominance in February and May is due to molluscs and amphipods, respectively: a key factor is the different trophic specialization of the dominant species, i.e., *Gibbula ardens* and *Amphithoe helleri*, in relation to the specific epiphyte morphology present in each of the two seasons (encrusting vs. erect forms, respectively).

The explanation for the trends of the other trophic categories is less clear, apparently because of their intrinsic heterogeneity. The greater abundance of Carnivores at deep stations is due to the diversity of prey as well as to habitat stability and heterogeneity, which allows for a multiplicity of niches and interactions. Suspension feeders and Deposit feeders are linked to higher rates of deposition, which in turn are related to hydrological conditions in the water column overlying the deep portions of the stand.

Herbivore and Herbivore-deposit feeder guilds dominate the considered taxa, although different functional adaptations are conceivable in the individual taxonomic groups. This suggests that the grazing food chain is the main energy pathway at the leaf level. As epiphytes and associated detrital particles are the main food source for He and HeDF, their distribution is likely to be influenced
by seasonal and depth-related growth rhythms of both the epiphytes and the seagrass (Mazzella & Buita, 1989).

Some species, such as the polychaete Platynereis dumerilii, the molluscs Gibbula ardens, Rissoa italiensis, Bittium latreillii, the isopod Cymodoce hanseni, the amphipods Dexamine spinosa and Apherusa chiereghinii, as well as the decapods Hippolyte inermis and Cestopagurus timidus, play a key role in the community dynamics and in the food web at the leaf stratum level.

Summary

The vagile fauna of the Posidonia oceanica leaf stratum was studied at the level of main taxa such as polychaetes, molluscs, tanaids, isopods, amphipods, and decapods; a spatio-temporal analysis was performed on both taxonomic and trophic structures of the communities.

The investigations were carried out along a depth transect in a Lacco Ameno meadow (Island of Ischia, Italy) at five stations (1, 3, 10, 15, and 25m) and in four seasons (July, November, February, and May). The samples were collected by SCUBA divers using a hand-towed net.

The community, mainly composed of Mollusca and Amphipoda, showed almost the same patterns along the transect in all seasons: a shallow community, characterized by higher variability, and more stable intermediate and deep communities were identified. The trophic analysis revealed the strong dominance of Herbivores-deposit feeders at all depths and in all four months; it also revealed the presence of Herbivores at the shallow stations, mainly molluscs in February and amphipods in May. Carnivores and Deposit feeders-carnivores, mainly decapods, inhabited the deep stands. On the whole, the population structure was strongly coherent with the trophic structure.

Environmental gradients, coupled with biological adaptations of the main taxa (feeding habit), determined the observed pattern.

Herbivore and Herbivore-deposit feeder abundances were consistent with the distribution and growth rhythms of the plant and epiphytes. The grazing food chain, realized mainly via epiphytes (micro- and macroalgae) and particulate organic matter, is of paramount importance in the energy transfer at the level of Posidonia oceanica leaf stratum.

Acknowledgements

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Posidonia oceanica, structural and trophic analyses of vagile fauna


Chapter 3.3. Trophic guilds and energetics as a mean to investigate food webs

Summary

3.3.1. Decapod and algal associations from “Banco di Santa Croce” (a protected area in the bay of Naples): a key pathway in local food webs. (Cited by 6. I.F.=n.d.)
A new index is proposed to classify species in homogeneous feeding groups. Macrozoobenthic species sampled in Posidonia oceanica meadows are used to test the method and demonstrate the correctness of theoretical assumptions.

3.3.2. The use of feeding indices for the study of food webs: an application to a Posidonia oceanica ecosystem. (Cited by 11. I.F.=1.23)
The gut contents of fish in three Posidonia oceanica meadows off the island of Ischia (Bay of Naples, Italy) were investigated and numerical abundance of prey was recorded. Seasonal variations in the diets of fish, also at prey-species level, were demonstrated. The fish taxon plays essentially a macro-carnivore trophic role. In the investigated seagrass meadows the main trophic fluxes start from plant detritus, macrophyta, and microphyta (as primary producers) towards crustacean decapods, copepods, ostracods, and gammarid amphipods (as secondary producers) to fish. A low recycling rate (4%) within the fish community was observed. Larger fish predators (e.g., Sparidae), swimming over the leaf canopy, are the main exporters to adjacent coastal systems.

3.3.3. Diet of fish populations in Posidonia oceanica meadows off the island of Ischia (Gulf of Naples, Italy): assessment of spatial and seasonal variability. (Cited by 15. I.F.=1.42)
A close interaction among various disciplines is necessary for a comprehensive analysis of food webs. In this case gut contents were analysed using the feeding indices above mentioned (previous research) to find out the pathways of energy transfer from primary producers to the last consumers.
Abstract

The decapod and algal communities were investigated at the “Banco di Santa Croce”, a protected area in the Bay of Naples, Italy, mainly composed of rocky projections. Samples were collected monthly by an air-lift sampler at 4 stations, which consist of two ecological levels (photophilic and sciaphilic environments). The communities were dominated by few species of natantia (Eualus pusiolus, E. occultus, Athanas nitescens) and one species of reptantia (Cestopagurus timidus). Algae, foraminiferids, molluscs and crustaceans were demonstrated to be important food items in the diet of the dominant species of decapods. Since these species were frequently found in the gut contents of fish, we concluded that decapod crustaceans are important links between primary producers and the final consumers (exporters) in the food webs of the considered environments. These results may be crucial to optimise a correct program of protection and management for the protected area.

Key-words: decapod community, hard bottom, algae, food webs, fish, Mediterranean Sea.

Introduction

Several studies on decapod crustacean communities have been performed in the Mediterranean (Garcia Raso, 1990; Lopez de la Rosa & Garcia Raso, 1992), demonstrating some similarities in the species composition and in the community structures, despite the ecological differences characterizing various photophilic environments. Decapod crustaceans appear to adapt to the physical features of the inhabited biotopes (e.g., the development of rhizome layer in a Posidonia oceanica meadow, the existence of crevices and shelter, the algal cover and the level of sedimentation) and express the dominance of few species along a typical geometrical distribution in dominance-diversity curves (Ledoyer, 1984). In some environments decapod associations may reach high diversity (Abele, 1974). However, little is known about the quantitative structure of decapod communities in hard bottoms of the Mediterranean sea (Harmelin, 1964) and the ecological role of species, mainly the dominant ones, in structuring the community, is unknown. Previous studies demonstrated that the structure of decapod communities mainly depends on the physical and ecological characters of biotopes (shelter, predation, availability of shells for pagurids) while food resources are a less important factor, taking into account their large trophic adaptability (Zupo et al., 1989; Zupo & Fresi, 1985). Therefore, decapod communities are often characterized by high taxonomic diversity and low functional redundancy. This fact may explain why decapod communities are rich and diverse in vegetated environments, while they appear impoverished in soft bottoms. In fact, plants may represent for them both
shelter and an important food source, as several species are opportunistic omnivores (Zupo & Fresi, 1985) and may be considered as mesograzers. They are also considered to be an important link between the primary producers and the final consumers (Zupo & Nelson, 1999), although few experimental evidences support this assumption. In the present study, the decapod crustacean community from a calcareous hard bottom has been analysed, and compared to the algal assemblages at two depth levels (corresponding to photophilic and sciaphilic environments) to investigate the influence of different morpho-functional groups of algae on the diversity of animal associations and the role of algae, in various environmental conditions, in structuring the community. Moreover, the gut contents of fish and decapods were analysed, to assess the trophic role of decapods within the food webs of the considered environments.

Materials and methods

The “Banco di Santa Croce” is a sea-mount complex located in the eastern side (40°40’N; 14°26’E) of the Gulf of Naples (Italy) and it was recognized, since the last century (Colombo, 1887), for its high naturalistic interest, being the main rocky bank in this sector of the Bay of Naples. Sea-mounts have a calcareous composition and they arise from –60 m (detritic bottom) to about 11 m depth (top of the shallowest mounts), forming a quasi-circular structure. Previous studies (Ranzi, 1930; Russo, 1992; Bussotti s., 1999) indicated the general biological and ecological features of this area, characterized by strong hydrodynamic forces, a great amount of suspended particulate matter (POM) and a diverse plant community, which supports the development of rich and diversified animal associations. Two sites were established within the bank: the site A, with the top at 11 m depth, closer to the outflow of the Sarno River (impacting the system with freshwater containing various industrial pollutants), and the site B, standing about 70 m apart from site A, with the top at 16 m depth, protected by a strong current from the impacts of the Sarno river. Two sampling stations were established on each site, the first ones (A1, B1) on the top of mounts, the second ones (A2, B2) at –18 and –22 m, respectively. Typical photophilic algal communities characterized the shallow stations, while coralligenous associations characterized the deep stations, while coralligenous associations characterized the deep ones.

Monthly samples of benthic fauna were collected by an air-lift sampler in 40×40 quadrates. Samples were fixed in alcohol and, after sorting of decapods, each individual was identified at the species level and counted. The gut contents of the most abundant species were analysed by an optical microscope, after dissection of 15-20 individuals (when available). The abundance of each food item was evaluated (0, 25, 50, 75, 100% of the gut contents) and recorded. Samples of fish fauna were collected bimonthly, using a fix net. Each specimen was identified and its gut was dissected under an optical microscope, to identify the main food items, according to the method proposed by Zupo et al. (2001). Finally, photographs of algae were monthly taken in 4 fixed quadrats (20×20 cm) in each station. Algae were determined using a computerized system of image analysis and the algal cover for surface unit was computed for the main species. Algae were finally classified into functional groups (Steneck and Watling, 1982), according to their shape and toughness, to detect their influence on the structure of decapod communities (Tab. 1).
Tab. 1 - Main features of the considered functional groups of algae (AG) according to Steneck and Watling (1982).

 Principali caratteristiche dei gruppi funzionali algali (AG) considerati, secondo Steneck e Watling (1982).

- AG 2: Filamentous algae (Cladophora, Ectocarpus, Acrochaetium)
- AG 3: Foliose algae (Ulva, Porphyra)
- AG 4: Corticated macrophytes (Bryothamnion, Chondria, Acanthophora)
- AG 5: Leathery macrophytes (non calcareous-crustose)
- AG 6: Articulated calcareous algae (Halimeda, Corallina)
- AG 7: Crustose coralline algae (Lithothamnion, Lithophyllum)
- AG 9: Articulate calcareous algae (Corallina, Jania)
- AG 10: Epilithic encrusting calcareous algae (Lithothamnion)

A cluster analysis (complete linkage, Euclidean distance) was carried out to investigate relationships between the decapod populations and the plant morpho-functional groups. The main diversity indices were computed on the data collected, to analyse the community structure and compare it to the ones obtained in other environments. In particular, the following indices (Margalef, 1977) were applied to the considered populations:

- S: number of species
- D: numerical dominance \( (N_i/N_t)\% \)
- R: Margalef richness \( (S-1)/\ln N_t \)

where \( N_i \) = number of specimens of the species “i”; \( N_t \) = total number of individuals (all species).

Results

In total, 48 samples of benthic fauna and 3121 specimens of decapods were collected at the 4 stations, classified into 42 species (Tab. 2). The most abundant species were Cestopagurus timidus (850 individuals, pooling all samples), Eualus cf. pusiolus (581 ind.), E. occultus (356 ind.) and Athanas nitescens (559 ind.). The abundances of decapod species exhibited a typical geometrical distribution, with the former species followed by all the other, present with less than 100 individuals, pooling all samples (Fig. 1). The total abundance of decapod population, in the four stations, resumes the one of the 4 dominant species (Fig. 2a), with higher densities in the site B (both stations B1 and B2). In the “B” stations, no significant differences in the density of decapod population were observed between the shallow (B1) and the deep (B2) station. In contrast, the shallow “A” station (A1) was characterized by a higher density in respect to the deep (A2) station (Fig. 2). Various biodiversity indices (Fig. 2b) indicated an increase in the number of species moving from stations “A” to stations “B” and from shallow to deep environments. This increase reflects a similar trend in the Margalef diversity index (R; Fig. 2b). The total number of individuals, pooling all specimens in all samples, is strictly related to the total number of the 4 main species in the same stations. The dominance (Fig. 3) of the four main species was clearly demonstrated at all stations, although Cestopagurus timidus and Eualus occultus dominated the
Tab. 2 - Decapod species identified in the present investigation and total number of individuals collected (all samples pooled).

Specie di decapodi identificate nel presente lavoro e numero totale di individui raccolti (totale nei campioni).

<table>
<thead>
<tr>
<th>Specie</th>
<th>Total Nr. Ind.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Achaeus cranchii</td>
<td>34</td>
</tr>
<tr>
<td>2 Alpheus dentipes</td>
<td>106</td>
</tr>
<tr>
<td>3 Alpheus glaber</td>
<td>16</td>
</tr>
<tr>
<td>4 Alpheus macrocheles</td>
<td>3</td>
</tr>
<tr>
<td>5 Anapagurus breviaculeatus</td>
<td>3</td>
</tr>
<tr>
<td>6 Anapagurus laevis</td>
<td>53</td>
</tr>
<tr>
<td>7 Atelecyclus rotundatus</td>
<td>6</td>
</tr>
<tr>
<td>8 Athanas nitescens</td>
<td>559</td>
</tr>
<tr>
<td>9 Brachynotus sexdentetatus</td>
<td>3</td>
</tr>
<tr>
<td>10 Calcinus tubularis</td>
<td>56</td>
</tr>
<tr>
<td>11 Callianassa truncata</td>
<td>6</td>
</tr>
<tr>
<td>12 Cestopagurus timidus</td>
<td>830</td>
</tr>
<tr>
<td>13 Chibanarius erythropus</td>
<td>72</td>
</tr>
<tr>
<td>14 Ebalia cranchi</td>
<td>3</td>
</tr>
<tr>
<td>15 Ebalia edwardsi</td>
<td>6</td>
</tr>
<tr>
<td>16 Ebalia tumefacta</td>
<td>3</td>
</tr>
<tr>
<td>17 Entuba mascarone</td>
<td>9</td>
</tr>
<tr>
<td>18 Eualus occultus</td>
<td>356</td>
</tr>
<tr>
<td>19 Eualus pusiolus</td>
<td>581</td>
</tr>
<tr>
<td>20 Galathea bolivari</td>
<td>6</td>
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<tr>
<td>21 Galathea intermedia</td>
<td>3</td>
</tr>
<tr>
<td>22 Galathea nexa</td>
<td>3</td>
</tr>
<tr>
<td>23 Hippolyte garciarasoi</td>
<td>25</td>
</tr>
<tr>
<td>24 Hippolyte leptocerus</td>
<td>84</td>
</tr>
<tr>
<td>25 Hippolyte varians</td>
<td>72</td>
</tr>
<tr>
<td>26 Inachus phalangium</td>
<td>3</td>
</tr>
<tr>
<td>27 Lysmata seticaudata</td>
<td>3</td>
</tr>
<tr>
<td>28 Macropoda rostrata</td>
<td>3</td>
</tr>
<tr>
<td>29 Maja verrucosa</td>
<td>9</td>
</tr>
<tr>
<td>30 Munida intermedia</td>
<td>6</td>
</tr>
<tr>
<td>31 Palaemon longirostris</td>
<td>6</td>
</tr>
<tr>
<td>32 Palicurus caronii</td>
<td>19</td>
</tr>
<tr>
<td>33 Parthenope massena</td>
<td>6</td>
</tr>
<tr>
<td>34 Pilumnus hirtellus</td>
<td>9</td>
</tr>
<tr>
<td>35 Pisa armata</td>
<td>3</td>
</tr>
<tr>
<td>36 Pisa tetraodon</td>
<td>22</td>
</tr>
<tr>
<td>37 Portunus latipes</td>
<td>3</td>
</tr>
<tr>
<td>38 Processa canaliculata</td>
<td>6</td>
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<td>39 Sicypnia carinata</td>
<td>9</td>
</tr>
<tr>
<td>40 Spiropagurus elegans</td>
<td>9</td>
</tr>
<tr>
<td>41 Stenopus spinosus</td>
<td>6</td>
</tr>
<tr>
<td>42 Thoralus cranchii</td>
<td>75</td>
</tr>
<tr>
<td><strong>Tot.</strong></td>
<td><strong>3121</strong></td>
</tr>
</tbody>
</table>

The time trends of the decapod abundances were according to a complex pattern, largely variable in the four stations. Station A1 was characterized by an communities mainly in station A1, while Eualus cf. pusiolus and Athanas nitescens dominated the communities mainly in station B1. The deep stations were characterised by a higher diversity and a lower dominance of the main four species. Taking into account the dominance of the main species in the four stations, E. pusiolus mainly inhabited shallow stations; E. occultus and C. timidus mainly inhabited the “A” stations; A. nitescens preferred deeper and “B” stations, although it was present in all the considered environments.
Fig. 1 - Total number of individuals of the main species of decapods, obtained pooling all seasons and all stations.

Numero totale di individui delle principali specie di decapodi, ottenuto cumulando tutte le stagioni e tutte le stazioni.

Fig. 2 - a) Total number of individuals in the 4 stations, obtained pooling all yearly samples. 
b) Biodiversity indices computed for the 4 stations pooling all yearly samples. S=number of species; N=Number of individuals; R=Margalef index of species richness.

a) numero totale di individui nelle 4 stazioni ottenuto cumulando tutti i campioni di un anno. 
b) indici di biodiversità calcolati per le 4 stazioni cumulando tutti i campioni annuali. S=numero di specie; N=numero di individui; R=Indice di Margalef di ricchezza specifica.
Fig. 3 - Dominance \( (D=\text{Ni} \times \text{Nt} \times 100) \) of the main species in the four stations, obtained pooling all yearly samples.

*Dominanza \( (D=\text{Ni} \times \text{Nt} \times 100) \) delle principali specie di decapodi nelle 4 stazioni, ottenuta cumulando tutti i campioni annuali.*

increase in the total number of individuals in summer months (June, July, August) and a low abundance along the rest of the year. Station A2 exhibited an opposite trend, with maxima in winter (January, February) and a secondary peak in Sep-

Fig. 4 - a) Total cover of algal functional groups in the four stations (all yearly data pooled). b) Dominance of algal functional groups computed for the four stations (all yearly data pooled).

*a) Copertura totale dei singoli gruppi funzionali di alghe nelle 4 stazioni (tutti i dati cumulati).*

*b) Dominanza dei gruppi funzionali algali calcolata per le 4 stazioni (tutti i dati annuali cumulati).*
tember. Station B1 exhibited the highest increase of abundance in summer (July, August, September) and its population decreased during winter months. Station B2 exhibited a bimodal trend, with a summer increase (in July, August and September) and a winter peak (in January). These trends were largely influenced by the population sizes of the main species.

The algal cover was dominated by AG5 in station A1, A2 and B2, and by AG6 in station B1 (Fig. 4a). Station A1 also contained large abundances of AG7, AG4 and AG2 and AG3, while stations A2 and B2 contained large coverage of AG7 (Fig. 4b). Taking into account the distribution of algal groups along the stations (Fig. 4a,b), AG2 and AG3 were typical of shallow stations; AG4 were mainly shallow and more abundant in stations A; AG5 was deeper; AG6 was present only in B1 (shallow) station; AG7 were present in all environments but absent in B1 station. A linear relationship ($R^2=0.7$) was demonstrated between the algal cover (all functional groups pooled) and the decapod numerical abundance in the four stations (Fig. 5), with an increase from station A2 (lower cover, lower abundance of decapods) towards A1, B1, B2 (highest values of both decapod abundance and algal cover). In contrast, no significant relationship was found between the algal cover and the diversity of decapod associations in each station. The distribution of the main species of decapods may be partially explained by the presence of selected groups of algae (Fig. 6), according to the results of cluster analysis. A. nitescens and C. timidus were segregated into a single group, apparently being not influenced by the abundance of any algal group. E. occultus and E. cf. pusiolus, on the contrary, were linked to AG2, AG3, AG4 and AG6, respectively. AG5 was clustered in a separate group, being scarcely related to the abundance of any decapod species.

The gut contents of the main species of decapods indicated a large preference for plant materials. Macroalgae represented the 63% of the gut contents in C. timidus, the 14% in A. dentipes, the 37% in E. pusiolus, and the 28% in
E. occultus. Diatoms represented another important category in C. timidus (27% of the gut content) and E. pusiolus (21%). The diet of the main species contained, however, also animal items, the most important of them being acarids, other arthropods, molluscs, polychaets and foraminiferids.

Fig. 6 - Tree diagram obtained by cluster analysis on the matrix “decapod abundance/algal cover”.

Diagramma ottenuto mediante cluster analysis sulla matrice “Abbondanza dei decapodilcopertura algale”.

Fig. 7 - Gut contents of fish (all species pooled) collected in the same area and in the same experimental period, expressed as percent of the gut volume occupied by each food item, in summer and winter.

Contenuti stomacali dei pesci (tutte le specie cumulate) campionati nella stessa area e nello stesso periodo di studio, espressi come percentuale di volume occupato nella cavità gastrica da ciascuna categoria alimentare, in estate ed inverno.

In total 26 species of fish were sampled and submitted to gut content analyses: Anthias anthias, Apogon imberbis, Boops boops, Caecula imberbis, Chromis chro-
mis, Citharus linguatula, Coris julis, Gobius cruentatus, Mullus surmuletus, Pagellus erithrinus, Physcis phycis, Sardina pilchardus, Sarpa salpa, Scomber scombrus, Scorpaena notata, S. porcus, S. scrofa, Serranus cabrilla, S. hepatus, S. scriba, Solea ocellata, Spicara maena, Symphodus cinereus, S. tinca, Thalassoma pavo, Trachinus draco, Trachurus trachurus, Trigla lyra. The gut contents of these fish demonstrated, on the average, a high abundance of decapod crustaceans, besides molluscs, amphipods, larvae and various species of fish (Fig. 7). A higher presence of crustacean larvae and such decapod genera as Alpheus, Processa, Macropodia, Liocarcinus, Hippolyte, Galathea, Ebalia, Clibanarius, Cestopagurus were observed in summer gut contents (Fig. 7). In winter a higher diversity of decapod genera and a higher dominance of decapods within the other food items were found. The most abundant winter genera were Liocarcinus, Cestopagurus, Ebalia, Hippolyte, Processa, Palaeomon, Alpheus, but lower amounts of Eurynome, Galathea, Inachus, Palicus, Penaeus and Philocheras were also observed.

Conclusions

The studied hard bottoms exhibited high species diversity, comparable to the one demonstrated in other important coastal habitats (Abele, 1974), such as Posidonia oceanica meadows (Garcia Raso, 1990) and detritic bottoms. This observation reflects a high environmental richness and demonstrates the specific use of plant and animal resources by the decapod community. The trends of density and biodiversity observed along the four stations may be explained taking into account the abundances of the main species of decapods and their pattern of dominance in selected sites (Wolda, 1981). The general characteristics of the analysed environments are also according to “ecological zones” determined by two main abiotic influences: a) the topographical location (with the stations “A” closer to the influence of the Sarno river) and b) the depth gradient, determining a higher stability in the deep stations (A2, B2), also characterized by lower irradiance and hydrodynamics. In this view the data reported may be explained: a higher biodiversity is observed moving from stations B (less impacted) to stations A and from shallow to deep stations. In contrast, a higher density of specimens per surface unit is observed moving from shallow to deep stations (especially in the “A” stations) and from A to B (less impacted) stations. The algal cover exhibits an opposite depth pattern between the two (A and B) locations: higher covers were recorded from A2 (deep) to A1 and from B1 (shallow) to B2, although stations A (both shallow and deep) demonstrated, on the whole, higher cover than B stations.

The geometrical distribution in the abundance of species appears to be a typical feature of decapod associations (Ugland and Gray, 1982), as it has previously been observed in several different, environments (Lopez de la Rosa & Garcia Raso, 1992). It is interesting to observe that the main species (e.g., Cestopagurus timidus, Athanas nitescens, Eualus occultus, Alpheus dentipes, Hippolyte sp., Thoralus cranchii), are present in the whole Mediterranean basin and characterise most environments, as reported by previous authors (Garcia Raso & Fernandez Munoz, 1987; Garcia-Raso, 1990). However, the presence of species considered “rare” or absent in the Tyrrenian sea must be highlighted. It is worth observing the presence of several individuals of Palicus caronii, previously considered rare (besides Lewinsohn & Holthuis, 1986; d’Udekem d’Acoz, 1999). Moreover, Eualus cf. pusiolus was considered absent in the coastal waters of the western Mediterra-
Decapod food webs at Banco di Santa Croce

The relationships between decapod abundance and algal cover confirm their mesograzer habits and the importance of such plant groups as AG 2, AG3, AG5 and AG6. Algae, of course, play a major role in the food web of the decapod community and their abundance in the gut contents (both micro- and macroalgae) indicate that decapods are important consumers of the canopy, although their trophic adaptability (high diversity of plant and animal prey in the gut contents) is one of the features influencing the ecological success in all the coastal environments (Zupo & Fresi, 1985). The gut contents of fish demonstrated the importance of decapods as a food source for final consumers, both in summer and in winter. In particular, decapods contributed significantly to the diet of fish stocks in summer both with their larvae (meroplankton) and with various species standing with large densities in this season. In winter decapod larvae were less important in the gut contents of fish, and they were replaced by various adult individuals; as a result, the winter diversity of decapods in the gut contents of fish increased. These data demonstrate that decapod crustaceans represent a fundamental link between the primary producers and the final consumers in the food webs of the considered environments, promoting a direct transfer to the main exporters of the algal biomass produced into the bank.

Riassunto

Sono state studiate le comunità a crostacei decapodi presenti presso il Banco di Santa Croce, un’area a tutela biologica nel Golfo di Napoli, costituita da guglie rocciose. Sono stati raccolti campioni mensili mediante sorbona in 4 stazioni, che rappresentano 2 livelli ecologici (fotofilo e sciafilo). Sono stati ottenuti, in totale, 3121 individui, 42 specie. Le stazioni definite nella parte interna dell’area, meno disturbate, mostravano una maggiore biodiversità. Le comunità erano dominate da poche specie di natanti (Eualus pusioslus, E. occultus, Athanas nitescens) ed una specie di reptanti (Cestopagurus timidus). I trend stagionali delle abbondanze numeriche dell’intera popolazione di decapodi, caratterizzati da due picchi annuali (estate ed inverno), coincidono con quelli delle specie dominanti. La copertura algale mostra un andamento opposto: aumenta dalle stazioni profonde alle superficiali nella parte esterna del banco, e dalle superficiali alle profonde nella parte interna. Esiste peraltro un gradiente spaziale ed una marcata specificità nell’uso delle risorse trofiche da parte delle varie specie di decapodi. Alghe, foraminiferi, molluschi e crostacei sono stati dimostrati essere fonti alimentari importanti nella dieta delle specie dominanti di decapodi. Poiché queste stesse sono state identificate frequentemente nei contenuti stomacali dei pesci campionati, se ne conclude che i crostacei decapodi costituiscono un importante livello trofico tra produttori primari (alghe) e consumatori finali (pesci), in grado di esportare la produzione ad altri sistemi limitrofi.

Acknowledgements

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References


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THE USE OF FEEDING INDICES FOR THE STUDY OF FOOD WEBs: AN APPLICATION TO A POSIDONIA OCEANICA ECOSYSTEM

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Keywords: Food preferences, Gut contents, Multivariate analysis, Trophic groups.

Abstract. I propose the use of new feeding indices, to classify species in homogeneous trophic groups. To illustrate the approach I examined macrozoobenthic species sampled in Posidonia oceanica meadows off the Island of Ischia (Mediterranean Sea, Gulf of Naples).

Introduction

The study of the food web of a complex system, such as a Posidonia oceanica bed, involves many aspects. A crucial point is to understand the role of each species in the web (Ledoyer 1962); this is done by means of direct methods: analysis of gut contents, food preference studies, etc., or indirect methods: trophic groups’ analysis (Gambi et al., in press; Russo 1989, Fauchald and Jumars 1979, Parchon 1977).

Size and type (plant or animal) of preys are two important aspects for the interpretation of diet pattern (Fauchald and Jumars 1979). When multivariate analysis is applied to “gut content-species” matrices, the ordination of species in the space of the first significant axes reflects mainly the size and type (plant or animal) of preys (Chessa et al. 1983; Zupo and Freni 1984).

I propose trophic data be treated via a mathematical approach to study trophic data not only on the basis of multivariate methods, but also by means of two new indices that characterize species according to their feeding behaviour as defined by gut contents.

Materials and methods

a) Study area and data collection

The community data derived from samples collected on Posidonia oceanica beds around the Island of Ischia (Figure 1), where the meadows form a continuous belt from depths of 1 to about 33 m (Colantonio et al. 1982). Twelve samples were collected by means of a bottom trawl with a 4 cm mesh, in 12 random points of the meadow. Six samples (1 to 6) were collected in winter, the others (7 to 12) in summer (see Figure 1). All specimens of each sample were deep frozen to prevent digestion of the ingested preys, then fixed in 70 % alcohol, identified at species level (Table 1a) and dissected for the analysis of the gut content. Species were only considered if more than 5 individuals were sampled.

Gut contents were classified in 25 categories (“food items”) and the average gut content of each species was computed according to abundance, on the basis of an arbitrary code, ranging from 0 to 3. This produced a matrix of 77 species (consumers) and 25 food items (Table 1b).

b) Statistical analysis

The data matrix was analyzed by means of correspondence analysis (Legendre and Legendre 1984, Chessa et al. 1983), and by clustering techniques. Average linkage (Orlič 1978) was applied to the correlation matrix between food items, to obtain food items’ groups, while the sum of squares was applied to the distance matrix between consumers, to obtain trophic groups of species (Feoli and Feoli-Chiapella 1979). The analysis of redundancy and specific variance (Orlič 1973) was done for food items in order to weigh their importance in the data structure. This should also reflect their importance in the food web: if a food item has high redundancy it means that it is common in the diet of the considered species; if a food item has high specific variance it is independent from other preys.

The indices proposed, to order the species on the basis of prey-type and prey-size, respectively, are obtained from the following formulae:

Prey type index:

$$T_i = \left( \sum V_i - \sum C_i \right) / \sum M_i$$

Prey size index:

$$S_i = \ln \left( \sum (P_i S_i) \times M_i \right) / \sum M_i$$

where:

- \(V_i\) = abundance (or frequency) of vegetal items;
- \(C_i\) = abundance (or frequency) of animal items;
<table>
<thead>
<tr>
<th>N.</th>
<th>Species</th>
<th>N.</th>
<th>Species</th>
</tr>
</thead>
<tbody>
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<td>Ditrupa arietina</td>
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<td>Anapagurus sp.</td>
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<td>Eunice pennata</td>
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<td>Cibianarius erythropus</td>
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<tr>
<td>3</td>
<td>Hermonia hystrix</td>
<td>36</td>
<td>Doriipe lanata</td>
</tr>
<tr>
<td>4</td>
<td>Hyalinoecia tubicola</td>
<td>37</td>
<td>Ethusa mascarone</td>
</tr>
<tr>
<td>5</td>
<td>Lumbrineris latreilli</td>
<td>38</td>
<td>Eurynome aspera</td>
</tr>
<tr>
<td>6</td>
<td>Notomastus sp.</td>
<td>39</td>
<td>Inachus communissimus</td>
</tr>
<tr>
<td>7</td>
<td>Pomatoceros triqueter</td>
<td>40</td>
<td>Inachus dorsetensis</td>
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<td>8</td>
<td>Sabella fabrici</td>
<td>41</td>
<td>Inachus phalangium</td>
</tr>
<tr>
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<td>Serpula vermicularis</td>
<td>42</td>
<td>Inachus toracichus</td>
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<tr>
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<td>Spirographis spallanzani</td>
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<td>Palaeon xiphias</td>
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<td><strong>MOLLUSCA</strong></td>
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<td>Anomia ephippium</td>
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<td>Tellina balaustina</td>
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<td>Callostoma granulatum</td>
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<td>Pagurus alatus</td>
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<td>15</td>
<td>Calyptrae chinensis</td>
<td>48</td>
<td>Pagurus prideaux</td>
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<td>16</td>
<td>Glans trapetia</td>
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<td>Parthenope massena</td>
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<td>Chlamys varia</td>
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<td>Processa macroptalmata</td>
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<td>Denticula dentalis</td>
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<td>21</td>
<td>Fusinus syracusanus</td>
<td>53</td>
<td>Astropecten irregularis</td>
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<td>Gibbula ardens</td>
<td>54</td>
<td>Echinaster sepositus</td>
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<td>Jujubinus exasperatus</td>
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<td>Echinocardium mortenseni</td>
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<tr>
<td>24</td>
<td>Jujubinus striatus</td>
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<td>Hacelia attenuata</td>
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<td>25</td>
<td>Bulinus brandaris</td>
<td>57</td>
<td>Holothuria tubulosa</td>
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<td>26</td>
<td>Plagiocardium papillosum</td>
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<td>Luidia ciliaris</td>
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<td>Ophioderma longicaudum</td>
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<td>Tricholophus pullus</td>
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<td>Ophiomiex pentagona</td>
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<td>Tricholophus speciosa</td>
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<td>Ophiotrix quinquemaculata</td>
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<td>Venericardia antiquata</td>
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<td>Ophiura texturata</td>
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<td>Cymodoce truncata</td>
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<td>Paracentrotus lividus</td>
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<td>Psammochinus microtuberc.</td>
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<td><strong>PISCES</strong></td>
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<td>Arneglussius imperialis</td>
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<td>Sphaerechinus granularis</td>
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<td>69</td>
<td>Hippocampus ippocampus</td>
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<td>70</td>
<td>Hippocampus gutulatus</td>
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<td>71</td>
<td>Labrus bimaculatus</td>
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<td>72</td>
<td>Lepadogaster candidlei</td>
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<td>73</td>
<td>Scorpaena porcus</td>
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<td>Scorpaena scrofa</td>
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<td><strong>TUNICATA</strong></td>
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<tr>
<td>75</td>
<td>Serranellus hepatitis</td>
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<tr>
<td>76</td>
<td>Symphodus rostratus</td>
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<td><strong>TUNICATA</strong></td>
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Table 1b. Food items identified in the gut contents.

<table>
<thead>
<tr>
<th>N.</th>
<th>ITEM</th>
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<tbody>
<tr>
<td>1</td>
<td>Green Posidonia tissues</td>
</tr>
<tr>
<td>2</td>
<td>Brown Posidonia tissues</td>
</tr>
<tr>
<td>3</td>
<td>Macrophytes</td>
</tr>
<tr>
<td>4</td>
<td>Diatoms</td>
</tr>
<tr>
<td>5</td>
<td>Dinoflagellates</td>
</tr>
<tr>
<td>6</td>
<td>Radiolarians</td>
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<tr>
<td>7</td>
<td>Coccolithophorids</td>
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<tr>
<td>8</td>
<td>Foraminifers</td>
</tr>
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<td>9</td>
<td>Organic detritus</td>
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<tr>
<td>10</td>
<td>Bryozoans</td>
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<tr>
<td>11</td>
<td>Spongias</td>
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<td>12</td>
<td>Nematodes</td>
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<td>13</td>
<td>Sedentarian polychaetes</td>
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<tr>
<td>14</td>
<td>Errantia polychaetes</td>
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<td>15</td>
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<td>Halacarids</td>
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<tr>
<td>18</td>
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<tr>
<td>19</td>
<td>Isopods</td>
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<tr>
<td>20</td>
<td>Amphipods</td>
</tr>
<tr>
<td>21</td>
<td>Reptantia decapods</td>
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<tr>
<td>22</td>
<td>Natantia decapods</td>
</tr>
<tr>
<td>23</td>
<td>Other crustaceans</td>
</tr>
<tr>
<td>24</td>
<td>Holothuroids</td>
</tr>
<tr>
<td>25</td>
<td>Other echinoderms</td>
</tr>
</tbody>
</table>

$M_i = \text{abundance (or frequency) of each considered item;}$

$PS_i = \text{mean prey size (measured in mm or mg)}.$

The first index ($T$) defines prey type (plant or animal) and varies between -1 and 1: negative values identify carnivorous species, values close to 0 are typical of omnivores and positive values indicate plant feeding species. The second index ($S$) depends on prey size and has no limits; it is 0 when the mean prey size equals 1 mm, representing the limit between micro- and macrobenthos (McIntyre et al. 1984). Natural logarithm is used to discriminate microphages from others: since their prey are, on an average, smaller than 1 mm, the S index is negative.

As shown in Figure 2, in plots representing the ordination of species based on the two indices, it can be predicted that "omnivorous-detritus feeders" species,

![Figure 2](image)

Figure 2. Predictive model of trophic groups in the plane described by $T$ and $S$ indices. Detritus feeder species that use both animal and vegetal preys of different size, will be ordered in the center; large herbivores will be in the first quadrant (large plant materials); carnivorous predators in the second (large animal preys); micro-carnivores in the third (small animal preys); micro-herbivores (as grazers) will be confined to the fourth quadrant (small plant materials).

![Figure 1](image)

Figure 1. Sampling stations, in Posidonia oceanica prairies around the Island of Ischia. Samples from 1 to 6 were taken in summer, the others in winter.
that use both animal and vegetal preys of various sizes, will occupy a central position, while carnivorous predators (large animal preys) will be confined to the second quadrant, carnivorous microphagous (small animal preys), to the third, grazers, scrapers etc. (small plant foods) in the fourth, and large herbivores (large plant foods) in the first.

Differences in the indices obtained for a species, examined in space or time scale, can show adaptations to environmental conditions.

Ellipses of equal concentration (Lagonegro and Feoli 1985) based on the trophic groups revealed by the cluster analysis, were superimposed both on the ordination given by the two T and S indices and on that obtained by correspondence analysis.

Canonical correlation analysis was applied to correlate the first two axes obtained by correspondence analysis to the T and S indices, to verify the correlation of the two variable sets. Concentration analysis was applied to the "species-food items" matrix, after restructuration on the basis of the cluster analysis (applied to the food items and to the T and S indices), to define homogeneous trophic groups by means of the new indices. Trophic groups obtained by means of T and S indices were then used to analyze the food web of the P. oceanica prairies under study and to obtain a seasonal model.

**Results**

**a) Ecological observations**

A total of 841 specimens belonging to 76 species were collected (Table 1a). The population is dominated by echinoderms (39%), decapod crustaceans (27%) and molluscs (22%). Other taxa (polychaetes, amphipods, tunicates and fishes) accounted only for 12% of the total number of individuals. Species of both the foliar stratum and rhizome layer were found in all samples.

Gut contents were grouped in 25 food items, as shown in Table 1b. All the considered food items have a shared variance higher than 0.1; plant items show the highest specific variance and redundancy. The highest values of redundancy and specific variance are shown by *Posidonia* detritus, micro- and macro-Epiphytes (Dia, Mac) and Crustaceans, which can be considered the main pathways of energy transfer in the system (Figure 3).

As shown in Figure 4, cluster analysis, applied to the gut contents, distinguished two main groups of food items: the first consisting of plants and associated epibiotic community (green and brown *Posidonia* tissues, macroalgae, diatoms, sponges, radiolarians, foraminifers, etc.), and the second represented principally by secondary producers (polychaetes, crustaceans, echinoderms, fishes). Within the first cluster, plants by epiphytes are broken down further.

Cluster analysis applied to consumer species (Figure 5) defines three main trophic groups: the first contains herbivore species, the second mainly fishes and other macro-carnivores, the third mainly detritus feeders and omnivores.

The results of correspondence analysis applied to the "species-food items" matrix are shown in Figure 6. Three clusters were identified, and they corresponded to the main trophic groups derived by the cluster.
Figure 4. Dendrogram of food items obtained by hierarchical classification of food items, by average linkage clustering applied to the correlation matrix. See Table 1b for identification of food items number.

Figure 5. Dendrogram of the species grouped by the sum of squares clustering based on geodesic distance. Numbers refer to species indicated in Table 1a. A = herbivores; B = microherbivores; C = omnivores and detritus feeders; D = Carnivores; E = opportunistic herbivores.
Figure 6. Ordination of the species obtained by the correspondence analysis performed on the "species-food items" matrix. Ellipses of equal concentration are superimposed. He = herbivores; Cp = carnivores; DeF = detritus feeders and omnivores.

Moreover, ellipses of equal concentration were superimposed on these groups. Data were mainly ordered, on the first axis, on the basis of the prey type (plant or animal) and, on the second, on the basis of prey size.

b) Indices

T and S indices were calculated for each species (see Table 2). The ordination given by the S and T indices (represented, respectively, on the axes 1 and 2) is shown in Figure 7. Ellipses of equal concentration based on the three main trophic groups were superimposed also in this case. The distance between the centroids of ellipses is significant with both procedures, although some groups seem to overlap. Cluster "He" contains most of the micro-herbivores species sampled, while cluster "Cp" contains large predators and "DeF" the detritivorous species, according to the predictive model reported in Figure 2. Also the pattern of clusters is similar in the representations obtained with the correspondence analysis and with the feeding indices, with the cluster "DeF" partially overlapping "He" and "Cp".

Canonical correlation analysis showed that 84.78% of variance is common between the two first axes obtained by the correspondence analysis and the T and S axes. According to the Bartlett (1974) test, the hypothesis (independence between the two types of representation) can be rejected at p = .01.

The concentration analysis applied to the "trophic groups-food items" matrix (Figure 8) shows the correspondence between food items and the trophic groups identified by the T and S indices: carnivores are segregated in the cluster containing large vagile organisms and Crustaceans; omnivores and opportunistic herbivores are related to small vagile organisms, plant materials and other animal preys; mesograzers and microherbivores are found in the second and third quadrant, in relation to plant matter and epiphytes.

The numerical limits of the T and S indices that define the trophic groups obtained by this analysis can then be used to define trophic groups in the T, S plane. When we plot the areas defined by these limits on a cartesian system (Figure 9) we can verify the correspondence with the provisional model shown in Figure 2. We therefore suggest that the limits reported in Figure 10 can be used to define trophic groups of species on the basis of the proposed feeding indices (Table 2).

Figure 7. Ordination of the species in the T (type) and S (size) plane. Ellipses of equal concentration are superimposed. He = herbivore; Cp = carnivores; DeF = detritus feeders and omnivores.

Figure 8. Concentration analysis of the "trophic groups-food items" matrix. mHe = micro-herbivores; Meso = mesograzers; HeOp = opportunistic herbivores; DeF = detritus feeders and omnivores; Cp = carnivores; Plant = plant materials; 1VF = small vagile fauna; LVF = large vagile fauna; Cruc = crustaceans; AnEpif = animal epiphytes; Oth = other animals (holothuroids, bryozoans, etc.).
Figure 9. Ordination of species by the T and S indices. The boxed areas represent the groups defined by cluster analysis (A = herbivores; B = micro-herbivores; C = omnivores; D = carnivores; E = opportunistic herbivores). Circles represent the limits proposed to define trophic groups.

Figure 10. Proposed limits for the T and S indices characterizing each trophic group (abbreviations as in Table 2).
Table 2. Species ordered in trophic groups by means of T (type) and S (size) indices. CoP = opportunistic carnivores; CP = macrophage carnivores; DeF = omnivores and detritus feeders; HeOp = opportunistic herbivores; mHe = micro-herbivores.

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c) Analysis of the Posidonia ecosystem

Figure 11 (A and B) shows the results of a trophic analysis of the system (by P. C. A.) performed on the trophic groups defined as described above on the "stations vs trophic groups" matrix. We observe (Figure 11A) that micro-herbivore species (mHe) are well defined, due to their numerical abundance, while carnivorous predators (CP) are close to such "euryphagous" species as opportunistic (CoP, HeOp) and detritus feeder (DeF) organisms. The ordination of the station points (Figure 11B) clearly separates the stations around "Punta Caruso" from those around "Punta Castello" along F2, while stations 1, 2 and 3 are clearly separated from the others along F1. The food web of the "Castello" area is based mainly on detritus feeders, while that of "Punta Caruso" is characterized by a higher abundance of micro-herbivores. The stations at the lower limit are characterized by a high number of individuals. The composition of the feeding groups of each station is variable, being related to such environmental conditions as depth and structural characteristics of the meadow, although summer samples are characterized by a high abundance of micro-herbivore species (Figure 12), and detritus feeders are more abundant in winter samples. Herbivore-opportunistic species are abundant throughout the year, while carnivore-opportunistic species are more abundant in winter months.

Discussion

Cluster analysis (Figure 4) clearly shows the main criteria of food selection: the main clusters indicate
that species choose their foods principally on the basis of type ("plant related" or "animal"), while sub-clusters give a size discrimination. This analysis also shows a link between of Posidonia epiphytes and the plant tissues. This is probably because most part of the macrozoobenthic species considered in this study can eat brown Posidonia tissues, but they do not select plant materials from the associated epiphytic community. There is clearly an absence of herbivore utilizing plant tissues directly; large amounts of Posidonia biomass flow in the food web through the detritus chain, consisting of several "opportunistic-detritus feeder" species (Figure 9). The high specific variance and redundancy of plant items (Figure 3) confirm the importance of primary production (mainly Posidonia detritus and plant epiphytes) in the food web and shows that detritus chain and grazing activity are the main pathways of energy transfer to the secondary production.

The three groups identified in Figure 7 are well defined. Carnivore predators, including many fishes,

Figure 11. Principal component ordination of the "stations-trophic groups" matrix, after the definition of groups by means of the T and S indices. A: Trophic groups ordination (MHe = micro-herbivores; CP = carnivores; COP = opportunistic carnivores; HeOp = opportunistic herbivores; DeF = detritus feeders and omnivores); B: station ordination.

Figure 12. Trophic composition in summer and winter by summing data from all the stations. Abbreviations as in Table 2.
are present in the second quadrant; grazers, such as many species of molluscs, are in the fourth quadrant, close to some filter feeders and other micro-herbivore species; such detritus feeders as Holothuria tubulosa occupy a central position, as expected by the provisional model of Figure 2. Macro-herbivores and micro-carnivores are absent: according to the provisional model of Figure 2, these would be ordered, respectively, in the distal portions of the first and third quadrant.

By using the ellipses of equal concentration we also observe a high correspondence between the clusters obtained by correspondence analysis (Fig. 6) and those deriving from the scatterplot of the two indices (Figure 7), so that the two representations provide a single model of the meadow, in which the importance of grazers and detritus feeders is assessed (Figure 9). Also Canonical Correlation analysis shows high correlation between the two different representations.

In the PCA of the "stations-groups" matrix, the split along F2 (Figure 11b) can be due to the less developed leaf canopy that characterizes the meadows around Punta Caruso (in the eastern region of the island). In this area the meadows grow on rocky bottoms interrupted by patches of Cymodocea nodosa, rocks, and sandy sediments. This meadow is less extended and more exposed to external influences. Meadows in the second group (in the western region of the island) are larger, continuous, and characterized by a higher leaf canopy and density (Colantonio et al., 1982; Giraud et al., 1979).

Probably because of these differences, the stations of "Punta Caruso" are characterized by a higher number of microherbivore and a lower number of detritus feeders with respect to those of "Castello" area. In fact, in the less dense prairie, the litter accumulation can be at a lower scale, due to the continuous export of detritus by the currents. The separation of stations 1, 2 and 3 from the other stations, along F1, is probably due to the fact that samples were collected along the internal limit of the prairie.

The comparison of summer and winter samples, to study the seasonal trophic dynamics of the system, shows adaptations of the animal community to the different environmental conditions (Figure 12). In particular, the higher abundance of micro-herbivore (mHe) species in summer samples can be related to the plant growth and to the development of the epiphytic community, while the abundance of detritus feeders (DeF) in winter can be due to a higher litter biomass.

Conclusions

The main factors governing many trophic systems are prey type (plant or animal) and size. Utilizing two feeding indices it is possible to define each species on the basis of feeding behaviour, and to classify species in trophic groups, which can be utilized for further analysis.

The trophic indices described in the present study can be used to compare data resulting from different investigations and to define differences observed in the feeding behaviour of a species studied under temporal or spatial scales.

By means of homogeneous trophic groups based on T and S indices, it was shown that the food web of Posidonia oceanica is highly influenced by the presence of micro-herbivores and detritus feeder species, and plant materials seem to flow into the web mainly through these channels, due to the relative absence of pure macro-herbivores.

The abundance of litter can modify the structure of food webs of meadows that are differently exposed, thereby influencing the abundance and composition of the important "detritus chain" (Velimirov et al., 1981; Ott and Maurer 1977). The trophic groups assemblage varies according to the structural characters of the meadows, such as exposure, density of the beds, etc. This phenomenon is even more obvious when different areas of the same bed are considered. In fact, the internal limit zones are characterized by a higher density of "detritus feeders" and of "opportunist carnivores".

The number of microherbivores is higher in summer, while in winter there is an increase of detritus feeders. This result can be attributed to a higher amount of litter in winter months and to a more developed epiphytic layer in summer months.

The data reported here are preliminary and further studies are needed to understand better the results described. However, the findings confirm that the proposed indices can be useful to standardize feeding groups; in addition they can be utilized for statistical analysis, they allow comparisons of results by different authors, and contribute to our understanding of the complex systems food webs.

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REFERENCES


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SYN-TAX 5.0

A new, extended and completely revised version of a program package designed by J. Podani (L. Eötvös University, Budapest, E-mail: PODANI@LUDENS.ELTE.HU) for exploring multivariate data structures.

Main features: a unique user-friendly interactive environment with pictorial and text menus, parameter windows and on-line help; easy access to DOS and Utilities; useful also in batch mode in form of more than 50 stand-alone applications.

Classification: hierarchical clustering (22 different divisive and agglomerative strategies, 40 distance and dissimilarity functions, coefficients for mixed data, generalized distances); non-hierarchical classification (k-means clustering, quick clustering of very large data sets, global optimization, multiple partitioning); minimum spanning trees; rearrangement of data matrices via block clustering; fuzzy clustering.

Ordination and related methods: principal components analysis, correspondence analysis; metric and nonmetric multidimensional scaling; seriation (optimization of diagonal structure in data and distance matrices); canonical correlation analysis; canonical variates (discriminant) analysis; eigenanalysis.

Evaluation of classifications and ordinations: comparison of distance matrices, dendrograms, partitions, fuzzy partitions or ordinations; consensus partitions; fuzzy consensus methods, Procrustes analysis for consensus ordinations; significance test of matrix, dendrogram, partition and ordination dissimilarities via Monte Carlo simulations of distributions; multiple comparisons; importance of variables in clustering; probability ellipses to enhance ordination interpretability, cophenetic correlation.

Character ranking: ordering of variables according to their contribution do data structures in terms of correlation, covariance and information theory measures.

Analysis of multispecies point patterns: computer simulated sampling from digitized patterns; pattern detection in species assemblages.

Graphics: dendrograms, ordinations (simple scattergrams, biplots, canonical variates analysis plots indicating group memberships, superimposed minimum spanning trees and partitions, etc.), probability ellipses, matrix comparisons, rotating plots (a great method to create the illusion of three dimensions); minimum spanning trees, point patterns, histograms and line diagrams. PCX and TIFF saved screen images.

Utilities: data standardization and transformation; data entry and editing; conversion of data formats; flexible shortest path adjustment of ecological distance matrices.

System requirements: IBM-pc or compatibles with DOS 3.0 and higher (icons provided for WINDOWS support); CGA, Hercules, EGA or VGA monitor for graphics; hard disk; mathematical co-processor not required but used if present; min 520 Kbytes of free RAM; 300 Kbytes of EMS recommended for accelerating the user interface. Direct support of HP Laserjets and Epson 24-pin dot-matrix printers and compatibles.

Availability: the package may be obtained from SCIENTIA Publishing and Software Marketing, P.O. Box 658, H-1365 Budapest, Hungary. Cost: 250 US$ (educational), 350 US$ (network and non-educational). Upgrade: 90 US$. Write for a free DEMO diskette!
Diet of fish populations in posidonia oceanica meadows off the Island of Ischia (Gulf of Naples, Italy): assessment of spatial and seasonal variability

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ABSTRACT

The gut contents of fish in three Posidonia oceanica meadows off the island of Ischia (Bay of Naples, Italy) were investigated. A total of 926 individual fish belonging to 28 species was sampled by bottom trawl in the leaf canopy. Labridae, Pomacentridae, Scorpaenidae, and Serranidae were the best represented families (41%, 38%, 8% and 6% of the total number of individuals, respectively). Of the 94 taxa detected in the gut contents, 42 were identified to the species level. The most common food items were decapod crustaceans (15% of the gut contents, on average), copepods (13%), amphipods (14%), brown fragments of P. oceanica (6%), and ostracods (6%). The most abundant species of labridae, Symphodus ocellatus and S. rostratus, showed a broad spectrum of prey. This generalist feeding may positively influence their numerical abundance. Seasonal variations in the diets of fish, also at prey-species level, were demonstrated. The fish taxon plays essentially a macro-carnivore trophic role. In the investigated seagrass meadows the main trophic fluxes start from plant detritus, macrophyta, and microphyta (as primary producers) towards crustacean decapods, copepods, ostracods, and gammarid amphipods (as secondary producers) to fish. A low recycling rate (4%) within the fish community was observed. Larger fish predators (e.g., Sparidae), swimming over the leaf canopy, are the main exporters to adjacent coastal systems.

Keywords: Fish; Food Webs; Spatial Variability; Posidonia Oceanica; Seagrass; Seasonality

1. INTRODUCTION

The presence of seagrasses provides effective protection against predation [1-3]; therefore it is accompanied by a great abundance of small invertebrates [4-6]. In fact, the 3-dimensional complex structure provided by seagrasses represents a clear advantage for several invertebrates, as well as for young fish, that find refuge from predation [7]. It has been demonstrated that the capture success of predators is generally higher over bare substrates than in seagrass meadows and this leads settling larvae, juveniles and adults towards coastal meadows [8]. A rich fish population inhabits the seagrass meadows, because it is attracted to the abundant food (i.e., small invertebrates; [9]) and to the shelter from predators typically provided by these structured habitats. In fact, seagrass meadows play the role of nurseries for important fish species [10,11] that, along with decapod crustaceans [12,13], are important consumers of secondary production in these systems [14-16]. According to [7], the relative value of seagrasses as predation defense is correlated to the relative abundance of ambush-, stalk- and chase-attack predators inhabiting seagrass and neighboring substrata.

Several authors have investigated the structure of the food webs in Posidonia oceanica meadows (e.g., [5,17-19]). However, there is still a remarkable lack of information on the main pathways of transfer from the plant level to the highest trophic levels [20,21]. In particular, the fate of secondary production in the food webs of P. oceanica meadows is partially unknown. Although fish are hypothesized to be the highest level consumers of secondary production in seagrass meadows [22,23] and in other environments [24], the rate and the pathways of transfer are still uncertain.

Another important feature of Mediterranean sea-
The feeding behaviour of fish living within the leaf canopy of three Por. oceania meadows has been investigated in the present paper, through the analysis of their gut contents, to assess their role in the consumption of secondary production in two seasons and, therefore, the impact of fish predation [28] on invertebrate populations. Our major questions were: 1) Which is the trophic role played by fish in a range of Por. oceania meadows? 2) Are the trophic guilds exhibited by selected species of fish stable and time-persistent, but their canopy exhibits important seasonal variations due to the characteristic rhythm of growth [17]. These variations are also in accordance with dramatic shifts in the amount of detritus and epiphytes available [25,26]. In addition, seasonal differences in abundance and composition of associated invertebrate populations were observed in the leaf stratum [27]. Similar variations may be observed among differently exposed meadows. In fact, Por. oceania beds located in areas influenced by high hydrodynamic pressure exhibit lower abundance of detritus and different epiphyte associations, as compared to meadows exposed to low hydrodynamic forces [2,5]. Due to these spatial and temporal differences in the abundance of potential prey, the general assumption that fish represent important predators for selected invertebrates living in the leaf stratum of seagrasses [1,14,16] should be confirmed by direct data.

The feeding behaviour of fish living within the leaf canopy of three Por. oceania meadows has been investigated in the present paper, through the analysis of their gut contents, to assess their role in the consumption of secondary production in two seasons and, therefore, the impact of fish predation [28] on invertebrate populations. Our major questions were: 1) Which is the trophic role played by fish in a range of Por. oceania meadows? 2) Are the trophic guilds exhibited by selected species of fish stable and time-persistent, but their canopy exhibits important seasonal variations due to the characteristic rhythm of growth [17]. These variations are also in accordance with dramatic shifts in the amount of detritus and epiphytes available [25,26]. In addition, seasonal differences in abundance and composition of associated invertebrate populations were observed in the leaf stratum [27]. Similar variations may be observed among differently exposed meadows. In fact, Por. oceania beds located in areas influenced by high hydrodynamic pressure exhibit lower abundance of detritus and different epiphyte associations, as compared to meadows exposed to low hydrodynamic forces [2,5]. Due to these spatial and temporal differences in the abundance of potential prey, the general assumption that fish represent important predators for selected invertebrates living in the leaf stratum of seagrasses [1,14,16] should be confirmed by direct data.

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species were analysed for variation in their food sources as a function of the sampling site and season. Gut content data were analysed by the technique described by [18], to classify species in homogeneous trophic groups and obtain an ordination of fish trophic groups according to the site and the season of sampling [27]. The technique involves the calculation, for each species in each sample, of two indices defining a trophic category, based on average prey “type” (plant or animal) and “size” (taking into account the average size in millimetres of each prey item). In particular, the two indices were obtained by the following formulae:

\[ Type_i = \left( \sum V_i - \sum C_i \right) / \sum M_{ij} \]  

\[ Size_i = \ln(\sum PS_j \times M_{ij} / \sum M_{ij}) \]

with:
- \( V_i \) = abundance of plant items;
- \( C_i \) = abundance of animal items;
- \( C_i \) = abundance of each considered item;
- \( M_{ij} \) = mean prey size (measured in mm).

This technique allows for an ordination of species in Cartesian plots showing feeding preferences, to compare the results obtained at different sites or during different seasons, and to simplify the understanding of complex ecosystem food webs. In fact, the positions of species in the 4 quarters of the plot indicate their feeding habits: macro-herbivores are ordered in the 1\(^{st}\) sector (upper right), micro-herbivores in the 2\(^{nd}\) sector (lower right), micro-carnivores in the 3\(^{rd}\) sector (lower left), macro-carnivores in the 4\(^{th}\) sector (upper left), and omnivores are close to the centre of axes. The information collected was used to draw the main trophic relationships in the considered system.

3. RESULTS

3.1. Fish Populations

No significant differences between the four replicates of each sample were found (ANOVA; \( p<0.01 \)). Therefore, the specimens collected in each set of four parallel replicates were pooled, prior to be subjected to trophic analyses. A total of 926 individual fish, belonging to 28 species (Table 1), was sampled, identified, and analysed. The most abundant families were Labridae (41% of all individuals), Pomacentridae (39%), Scorpaenidae (8%) and Serranidae (6%). The most abundant species (Figure 2) were Chromis chromis (356 ind.; 38%); Symphodus ocellatus (187 ind.; 20%) and S. rostratus (134 ind.; 14%). The total number of species and individuals in each sample varied according to the site and the season and it was highest in Lacco Ameno in summer (Figure 3).

The total number of species per sample varied between 7 (at the Procida channel, summer) and 16 (at Lacco Ameno, summer). The biomass of species sampled in winter was constantly higher than in summer, with the exception of the families Pomacentridae and Congeridae. Labridae reached a winter biomass of about 2 g m\(^{-2}\) (fresh weight) in the sampled meadows. The families Labridae, Pomacentridae and Serranidae accounted for 98% of the total fish biomass. Most individuals were small in comparison to the maximum size reached by the species (Table 1). Large intra-specific size variations were observed mainly in the samples collected at Lacco Ameno.

3.2. Analysis of Gut Contents

Ninety-four food items were identified in the guts.
Table 1. Fish species collected, total number of individuals collected in all the samples (pooled), mean length (cm total length) and mean weight (g wet weight) of each species.

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Species</th>
<th>Total nr. ind.</th>
<th>Mean length (cm)</th>
<th>Mean weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chromis chromis (Linnaeus, 1758)</td>
<td>356</td>
<td>5.43</td>
<td>7.37</td>
</tr>
<tr>
<td>2</td>
<td>Symphodus ocellatus Forsskal, 1775</td>
<td>187</td>
<td>6.04</td>
<td>1.07</td>
</tr>
<tr>
<td>3</td>
<td>Symphodus rostratus (Block, 1797)</td>
<td>134</td>
<td>8.41</td>
<td>13.52</td>
</tr>
<tr>
<td>4</td>
<td>Scorpaena porcus Linnaeus, 1758</td>
<td>69</td>
<td>8.48</td>
<td>4.82</td>
</tr>
<tr>
<td>5</td>
<td>Serranus scriba (Linnaeus, 1758)</td>
<td>42</td>
<td>11.01</td>
<td>3.75</td>
</tr>
<tr>
<td>6</td>
<td>Symphodus mediterraneus (Linnaeus, 1758)</td>
<td>26</td>
<td>5.57</td>
<td>4.65</td>
</tr>
<tr>
<td>7</td>
<td>Nerophis maculatus Rafinesque, 1810</td>
<td>17</td>
<td>12.48</td>
<td>1.03</td>
</tr>
<tr>
<td>8</td>
<td>Symphodus tinca (Linnaeus, 1758)</td>
<td>17</td>
<td>12.48</td>
<td>0.99</td>
</tr>
<tr>
<td>9</td>
<td>Gobius crucatus Gmelin, 1789</td>
<td>15</td>
<td>8.08</td>
<td>3.52</td>
</tr>
<tr>
<td>10</td>
<td>Serranus hepatus (Linnaeus, 1758)</td>
<td>12</td>
<td>6.22</td>
<td>10.16</td>
</tr>
<tr>
<td>11</td>
<td>Syngnathus acus (Linnaeus, 1758)</td>
<td>8</td>
<td>20.76</td>
<td>0.76</td>
</tr>
<tr>
<td>12</td>
<td>Arnoglossus kessleri Schmidt, 1915</td>
<td>6</td>
<td>5.00</td>
<td>2.78</td>
</tr>
<tr>
<td>13</td>
<td>Symphodus cinereus (Bonneterre, 1788)</td>
<td>6</td>
<td>7.73</td>
<td>67.20</td>
</tr>
<tr>
<td>14</td>
<td>Scorpaena notata Rafinesque, 1810</td>
<td>5</td>
<td>8.43</td>
<td>0.99</td>
</tr>
<tr>
<td>15</td>
<td>Apogon imberbis (Linnaeus, 1758)</td>
<td>4</td>
<td>8.08</td>
<td>3.52</td>
</tr>
<tr>
<td>16</td>
<td>Gobius geniporus Valenciennes, 1837</td>
<td>3</td>
<td>4.75</td>
<td>0.96</td>
</tr>
<tr>
<td>17</td>
<td>Labrus viridis (Linnaeus, 1758)</td>
<td>3</td>
<td>13.65</td>
<td>35.31</td>
</tr>
<tr>
<td>18</td>
<td>Mullus surmuletus (Linnaeus, 1758)</td>
<td>3</td>
<td>7.40</td>
<td>4.04</td>
</tr>
<tr>
<td>19</td>
<td>Conger conger (Linnaeus, 1758)</td>
<td>2</td>
<td>24.05</td>
<td>12.41</td>
</tr>
<tr>
<td>20</td>
<td>Diplodus annularis (Linnaeus, 1758)</td>
<td>2</td>
<td>12.60</td>
<td>3.59</td>
</tr>
<tr>
<td>21</td>
<td>Serranus cabrilla (Linnaeus, 1758)</td>
<td>2</td>
<td>10.01</td>
<td>18.83</td>
</tr>
<tr>
<td>22</td>
<td>Bothus podas (Delaroche, 1809)</td>
<td>1</td>
<td>4.80</td>
<td>1.27</td>
</tr>
<tr>
<td>23</td>
<td>Coris julis (Linnaeus, 1758)</td>
<td>1</td>
<td>8.80</td>
<td>20.87</td>
</tr>
<tr>
<td>24</td>
<td>Deltentosteus quadrimaculatus (Valenciennes, 1837)</td>
<td>1</td>
<td>6.26</td>
<td>4.87</td>
</tr>
<tr>
<td>25</td>
<td>Gobius vittatus Vinciguerra, 1883</td>
<td>1</td>
<td>5.25</td>
<td>1.12</td>
</tr>
<tr>
<td>26</td>
<td>Spicara maena (Linnaeus, 1758)</td>
<td>1</td>
<td>10.80</td>
<td>14.51</td>
</tr>
<tr>
<td>27</td>
<td>Symphodus doderleni Jordan, 1891</td>
<td>1</td>
<td>3.90</td>
<td>31.37</td>
</tr>
<tr>
<td>28</td>
<td>Symphodus melanocercus (Risso, 1810)</td>
<td>1</td>
<td>4.80</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Scarcely abundant food items were pooled into larger taxa and a matrix containing 28 species of fish (Table 1) and 30 food items (Table 2) was obtained. The most abundant species, *Chromis chromis*, fed mainly on plankton items, besides molluscs and decapods. In contrast, the two most abundant species of Labridae, *Symphodus ocellatus* and *S. rostratus*, fed on a wide spectrum of food items shared with the whole fish population. Their diet profiles, however, were different, since *S. ocellatus* fed mainly on copepods and other crustaceans, while *S. rostratus* exhibited a wider spectrum of preferences, including plathelminthes, small crustaceans, natantia decapods, and *Posidonia* tissues. Another important species of Labridae, *Symphodus mediterraneus*, showed a narrower dietary spectrum (21 items) and fed preferentially on brown *Posidonia* tissues, copepods, reptantia decapods, and other animal items. *Serranus scriba*, *S. cabrilla* and *Scorpaena porcus*, among the other abundant species, fed preferentially on natantia and reptantia decapods, but they exhibited a wide dietary spectrum, including *Posidonia* tissues, amphipods, and other animal items.

The ordination of species according to the “Type” and “Size” indices [18] indicated that the fish community plays essentially a macro-carnivore trophic role (Figure 4): species were all ordered in the 4th sector, in a compact cluster. An exception was represented by *Coris julis* and *Spicara maena*, exhibiting a “microphagous” feeding pattern, and *Scorpaena notata* and *Symphodus cinereus*, clustered towards a position indicating herbivorous...
Table 2. Food items taken into account in the present investigation and their average abundance (% of gut contents) in all samples.

<table>
<thead>
<tr>
<th>Prey item</th>
<th>% abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Copepods</td>
<td>12.93</td>
</tr>
<tr>
<td>2) Gammarid amphipods</td>
<td>10.06</td>
</tr>
<tr>
<td>3) Natantia decapods</td>
<td>9.26</td>
</tr>
<tr>
<td>4) Unidentified animal tissues</td>
<td>8.57</td>
</tr>
<tr>
<td>5) Reptantia decapods</td>
<td>6.45</td>
</tr>
<tr>
<td>6) Brown tissues of Posidonia</td>
<td>6.37</td>
</tr>
<tr>
<td>7) Ostracods</td>
<td>6.23</td>
</tr>
<tr>
<td>8) Unidentified crustaceans</td>
<td>6.01</td>
</tr>
<tr>
<td>9) Isopods</td>
<td>4.29</td>
</tr>
<tr>
<td>10) Caprellid amphipods</td>
<td>3.93</td>
</tr>
<tr>
<td>11) Fish</td>
<td>3.81</td>
</tr>
<tr>
<td>12) Macroalgae</td>
<td>3.19</td>
</tr>
<tr>
<td>13) Unidentified decapods</td>
<td>2.5</td>
</tr>
<tr>
<td>14) Plathelmintes</td>
<td>2.37</td>
</tr>
<tr>
<td>15) Mysidaceans</td>
<td>2.32</td>
</tr>
<tr>
<td>16) Nematodes</td>
<td>2.22</td>
</tr>
<tr>
<td>17) Tanaidacea</td>
<td>1.87</td>
</tr>
<tr>
<td>18) Polychaetes</td>
<td>1.54</td>
</tr>
<tr>
<td>19) Gastropod molluscs</td>
<td>1.12</td>
</tr>
<tr>
<td>20) Unidentified vegetal tissues</td>
<td>0.79</td>
</tr>
<tr>
<td>21) Eggs</td>
<td>0.78</td>
</tr>
<tr>
<td>22) Acarids</td>
<td>0.66</td>
</tr>
<tr>
<td>23) Foraminiferans</td>
<td>0.64</td>
</tr>
<tr>
<td>24) Microalgae</td>
<td>0.59</td>
</tr>
<tr>
<td>25) Sipunculids</td>
<td>0.4</td>
</tr>
<tr>
<td>26) Pantopods</td>
<td>0.4</td>
</tr>
<tr>
<td>27) Cumaceans</td>
<td>0.29</td>
</tr>
<tr>
<td>28) Unidentified molluscs</td>
<td>0.17</td>
</tr>
<tr>
<td>29) Bivalve molluscs</td>
<td>0.16</td>
</tr>
<tr>
<td>30) Echinoderms</td>
<td>0.06</td>
</tr>
</tbody>
</table>

feeding habit. *Chromis chromis* occupied a polar position, also indicating a microcarnivorous diet. The most abundant labridae, *S. ocellatus* and *S. rostratus*, were in a central position in the cluster. Besides the above exceptions, all the Scorpaenidae and Serranidae were grouped in a central compact sub-cluster.

Seasonal variations in the feeding habits of some species were observed (Figure 5). In summer *Labrus viridis* and *S. cinereus* exhibited a more “herbivorous” habit than in winter. In winter *S. ocellatus* preyed almost entirely on animals, while in summer it fed mainly on plant matter. *S. rostratus* did not change its feeding preferences (plant or animal) between the two seasons. No variations in the “Size” index were observed between the two seasons for any species, but the dietary composition changed. The total number of prey items of *S. ocellatus*
significantly changed according to the season. In fact *S. ocellatus* fed on 24 prey items in summer, and 20 in winter, when its diet was mainly based on copepods (32% of gut contents).

In contrast, the total number of prey items of *S. rostratus* was constant (24) in the two seasons and no significant differences were observed in the food composition.

Other species, such as *Serranus scriba* and *Scorpaena porcus*, exhibited a comparable reduction of plant items in winter, and different feeding preferences in the two seasons, as revealed by the “Type-Size” ordinations.

The samples obtained at Lacco Ameno (Figure 6(a)) and San Pancrazio (Figure 6(c)) clustered according to prey size, more tightly in respect to the Channel of Procida (Figure 6(b)). In particular, such species as *Mullus sur* 

![Figure 6. Ordination in the “Type-Size” space of the species according to the site of sampling (a, Lacco Ameno; b, Procida; c, S. Pancrazio). The horizontal axis discriminates the “type” of diet (based on the abundance of plant or animal prey items); the vertical axis discriminates the “size” of diet (based on small or large prey items). Circles indicate the position of each species of fish (the most abundant are specified).](image)

4. DISCUSSION

The skid trawl, as demonstrated by [29], is a suitable sampling tool to obtain a representative picture of the fish fauna living within the *Posidonia oceanica* meadows, allowing for the study of the upper levels of local food webs [30,31]. Other sampling methods, however, may complete the information on the fish assemblage of meadows, in particular on upper water dwellers, feeding mainly on planktonic micro-crustaceans or other fish [21,29]. The efficiency of the trawl was higher in winter, when the canopy was lower. The investigated meadows were characterised by benthic families of fish (Labridae, Pomacentridae, Scorpaenidae, Syngnathidae, Serranidae) although a few individuals were found that belonged to families with good swimming capabilities (e.g., Sparidae). The number of species collected during this investigation was lower than the number of species found in French meadows of *P. oceanica* meadows using an identical sampling technique (28 as compared to 49 species; [33]). The difference may be due to a higher fishing pressure characterising the meadows investigated in the present paper, in accordance with the results of previous studies [29].

Fresh weight estimates showed the importance of the main three families, i.e., Labridae, Pomacentridae and Scorpaenidae. In fact, they accounted for 99.4 % of the total fish biomass sampled in summer and for 93.7% of the total fish biomass sampled in winter (98% of the total fish biomass throughout the year).

The high fish biomass collected in winter was not correlated with individual fish weight. It was due to a
Table 3. Prey found in the gut contents of the most abundant species of fish collected. A grey square indicates the presence of each prey in summer (S) and/or winter (W) samples.

<table>
<thead>
<tr>
<th>Fish</th>
<th>S</th>
<th>W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apogon imberbis</td>
<td>Eualus occultus (Lebour, 1936)</td>
<td>Processa acutirostris Nouvel &amp; Holthuis, 1957</td>
</tr>
<tr>
<td>Chromis chromis</td>
<td>Hippolyte sp. Leach, 1814</td>
<td>Jujubinus sp. Monterosato, 1884</td>
</tr>
<tr>
<td></td>
<td>Liljeborgia dellavallei Stebbing, 1906</td>
<td>Siriella clausii G.O.Sars, 1876</td>
</tr>
<tr>
<td></td>
<td>Synisoma appendiculatum (Risso, 1816)</td>
<td></td>
</tr>
<tr>
<td>Scorpaena notata</td>
<td>Cymodoce hansenii Dumay, 1972</td>
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<td>1793</td>
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<td>Inachus thoracicus</td>
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<td>Munida intermedia A. Milne-Edwards &amp; Bouvier, 1899</td>
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<td>Pontogenia chrysocoma</td>
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<td>Syngnathus acus</td>
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<td>Anapagurus laevis</td>
<td>(Bell, 1846)</td>
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<td>Athanas nitescens</td>
<td>(Leach, 1814)</td>
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</table>

high numerical abundance; more individuals were caught in winter, probably due to the greater winter efficiency of the trawl in the lower canopy or to lower trophic resources of surrounding benthic systems [34]. Differences in the abundance of species at the three sites were demonstrated to be not significant, and several species were present with a low number of individuals and low biomass. Therefore we focused our investigation upon the most abundant species, i.e., the foremost 14 reported in Table 1. Most of the sampled fish species are carnivorous and should represent the top consumers within the meadow [7,33]. However, seasonal variations in the diet (plant or animal) of some species were detected in the present investigation.

The genus Symphodus was the most abundant, accounting for more than 40% of the total fish population.
Figure 7. Pathways of matter transfer (volumes of gut contents) in the fish food webs, based on the
data of the present paper (solid links between secondary producers and fish) and literature data
(dotted links between primary producers and secondary producers; see text). Only vertical links
were taken into account, to highlight the role of fish predation. The fish compartment is mainly
represented by Labridae, Pomacentridae, Scorpaenidae and Serranidae. Numbers in parenthesis indi-
cate the average volumes occupied by each item in the guts.

The diet of species in this genus was based on vagile organisms of the leaf stratum, grazers of the epiphyte
layer. Therefore the genus **Symphodus** represents one of the pathways from secondary producers within the leaf-
stratum to the export chain. The large spectrum of prey items found in the guts of the most abundant species, **Symphodus ocellatus** and **S. rostratus**, indicates high trophic adaptability, and this may be responsible for their success within the studied meadows, as documented by their abundance. In contrast, the diet of the most abundant species, **Chromis chromis**, was based on a few items, scarcely present in the guts of other fish. Therefore, this species has the advantage to use a rich, unexploited microphagous trophic niche. Other abundant species, such as **Scorpaena porcus** and **Serranus scriba**, fed almost exclusively on abundant [5] items in the meadow (e.g., decapod crustaceans and gammarid amphipods). **Symphodus rostratus**, **S. porcus** and **S. scriba** were efficient predators of decapod crustaceans, since decapods accounted for more than 30% of their gut volume. The abundance of these three species of fish may explain the high predation pressure observed on various decapod populations [35,36].

The diet of **S. ocellatus** and **S. mediterraneus**, in contrast, was based on smaller crustaceans (copepods and amphipods) accounting for more than 30% of their gut volume. The abundance of brown tissues of **Posidonia** in the guts of these species indicates that it is not an occasional item, although the actual trophic role of leaf detritus is unclear [37]. It could be used per se, or ingested to digest bacteria and small prey present on its surface [25,38,39].

The ordination of species in the “Type-Size” plots confirmed that fish play essentially a macro-carnivore role in the meadow food webs. Given the low rate of recycling (fish represented less than 4% of prey in the gut contents), it may be assumed that most of the biomass produced within the system is exported to other coastal systems through predation by fish swimming over the canopy, or lost through fishing activities [6]. The main variations were observed in the “Type” index, indicating that some species, such as **Symphodus cinereus** and **Labrus viridis**, can adapt their diets according to the availability of animal or plant items, while the average size of their prey did not vary. However, the diets of the most abundant species exhibited slight seasonal variations. The prey taxa consumed by most fish were abundant throughout the year [5], although variations of prey at a lower taxonomic level (i.e., species) were detected (Table 3).

The three dominant species of fish showed seasonal variations of prey at species level and some prey-species,
abundant in the gut contents in summer (such as *Hippolyte inermis*) were absent in winter (Table 3), according to their known [35] seasonal patterns of abundance in the meadows.

They were replaced in the diet by prey-species with a similar shape and size, such as *Eualus sp.* and *Processa acutirostris*. The seasonality in the prey availability was also demonstrated by the fact that such fish as *S. ocellatus*, *S. rostratus*, and *S. porcus* showed a larger diet spectrum in summer than in winter.

The ordination of species in the “Type-Size” space according to sites indicated that fish sampled off the island of Procida used prey characterised by a broader spectrum of sizes, as compared to the other two meadows. In fact, the maximum “Size” index reached by fish sampled at Lacco Ameno and San Pancrazio was about 1, while some species sampled at Procida, such as *Mullus surmuletus*, *S. scriba* and *S. porcus*, reached higher Size index scores. The comparable size of these fish species at the three sites suggests that differences shown in the Size index are dependent on prey availability.

A diagram of the main pathways of transfer, from primary producers to the top-level predators, can be drawn for the investigated *P. oceanica* meadows, based on the data of the present work and literature information on the feeding habits of the most abundant grazers. The guts contained mainly small invertebrates, typical of the leaf stratum, feeding on microphyta, macrophyta, and *Posidonia* detritus [12,40-44]. Taking into account the abundance (% of gut volume) of each food item in the two seasons, 12.9% of prey (in terms of gut volume) was represented by copepods, feeding, in their turn, mainly on diatoms and bacteria [34,41,45,46]. Gammarid amphipods accounted for 10.0% of fish prey and they feed mainly on micro-algae [47-51]. Reptantia decapods accounted for 6.4% of the fish prey and they feed mainly on *Posidonia* detritus and macrophyta [18], although horizontal links should be taken into account [52]. In fact, reptantia decapods also feed on other secondary producers, such as natantia decapods, amphipods, copepods, molluscs, tanaidacea, isopods, sipunculids, polychaetes [19,43,53,54]. Natantia deca pods accounted for 9.2% of the fish prey and they feed mainly on micro-algae and small organisms of the leaf stratum (amphipods, copepods, acarids; [5,35]), although horizontal links should be taken into account and also for this taxon [43,52,55,56]. Ostracods accounted for 6.2% of fish prey and they feed mainly on micro-algae [26,34]. Other small crustaceans accounted for 6.1% of the fish prey and they feed mainly on nematodes, copepods, and ciliates [57]. Isopods accounted for 4.3% of the fish prey and they mainly feed on micro-algae and detritus [58]. A low recycling rate in the fish compartment must be taken into account, since it was calculated that 3.8% of fish prey is represented by teleosts. Scorpaenidae, Serranidae, Pomacentridae and Congridae were the main fish feeders.

Our data indicated a pathway of transfer from *Posidonia* detritus, macrophyta and microphyta (as primary producers) to crustacean decapods, copepods, ostracods, and gammarid amphipods (as secondary producers) to fish (as consumers), mainly represented by Labridae, Pomacentridae, Scorpaenidae, and Serranidae. The rate of recycling through these families is low (about 4%). Crustacean decapods represent another important loop of biomass recycling, since they can feed also on the other secondary producers described above [14,18]. Therefore, besides natural mortality, the biomass stocked in the fish compartment (in terms of volumes) may be exported to other systems by means of predation by other fish [33], non resident in the meadows and characterised by higher swimming capabilities, such as Sparidae, larger Serranidae, and Congridae.

A comparison with studies in other seagrasses [10,13,20,59,60,61] and different sites of the Mediterranean [33] allows for detecting a general trend of fish assemblages, with respect to the feeding behaviour of dominant species. They generally show a clear preference for epibenthic fauna and extensively feed on crustaceans. Only few herbivorous and herbivorous-detritivorous species were detected in *P. oceanica* meadows, despite the large abundance of plant material available. In contrast, herbivorous and omnivorous fish are common in other seagrass communities [28,60,61,62], characterised by a larger variety of trophic levels. Most species were carnivorous, both macropaghic and microphagic, in accordance with the results obtained in French *P. oceanica* meadows [33]. The only herbivorous fish, well known in Mediterranean seagrass meadows, is *Sarpa salpa* (L.) [33]; however this species is generally restricted to shallow meadows (less than 10 m depth), characterised by a higher abundance of plant epiphytes. Therefore, it did not occur in the depth range investigated in the present paper and its feeding impact scarcely influences the food webs of deeper meadows, exhibiting a higher stability and complexity.

Labride are consistently dominant in Mediterranean *P. oceanica* meadows [22] and they feed on a broad spectrum of prey items, with a preference for crustaceans (mainly amphipods and decapods). However, they can adapt their diet according to the site and the time of sampling. In fact, our analyses indicated seasonal changes in the diet of some species and a lower abundance of molluscs in their gut contents, as compared to the results of [33]. Gastropod molluscs were mainly consumed by *Chromis chromis*, the most abundant species. In contrast, Labridae may be considered as mesophagic carnivores [33] feeding on copepods, gam-
marid amphipods, decapods, ostracods and other crustaceans, as well as molluscs. Decapods and other small crustaceans of the leaf stratum were demonstrated to be keystone items in the fish food webs [36], transferring biomass from the primary producers to the top predators. This trend appears to be a general feature of seagrass meadows [60,63], since it is in accordance with the results obtained in other seagrass ecosystems, both temperate and tropical.

5. ACKNOWLEDGEMENTS

We are indebted to P. Francour for the collection of samples in Ischia. We are grateful to M.C. Gambi, M.B. Scipione and M. Lorenti for their assistance in the determination of species. D. Stübing was supported by a COMETT fellowship, Europäischer Praktikantenaustausch. Mrs R. Messina revised the English text.

REFERENCES


Chapter 3.4. Determination of primary production and models

Summary

3.4.1. A production model for *Posidonia oceanica* based on temperature. (Cited by 41. I.F. = 2.61)
To produce feasible models of food webs a sufficient knowledge about the rates of primary production is indispensable, in order to define the initial budget of biomass entering the various trophic pathways. Since seagrasses are important models for coastal ecology and management, this study presents a model of production of the Mediterranean seagrass *Posidonia oceanica* based on weight. Temperature appears to be the main factor modulating biomass production during the year, while other factors, as light irradiance and nutrient availability are scarcely important to determine the seasonal growth rhythms.

3.4.2. Culture conditions influence the growth dynamics and the production of *Cocconeis scutellum* (Bacillariophyta). (Cited by 5. I.F. = 3)
Microalgae are also important primary producers and play a fundamental role both in planktonic and benthic environments. Their trophic role is comparable to the one of seagrasses for macrozoobenthic organisms, since they play both a trophic function and a structural function as first colonizers of benthic substrata. In addition, some diatoms are quite important because they produce secondary metabolites influencing the physiology of their consumers. The optimal conditions for the growth of two conspecific benthic diatoms were defined through factorial experimentation. The roles of light spectrum, nutrient availability, and culture conditions on the laboratory production of *Cocconeis scutellum scutellum* Ehrenb. and *C. scutellum parva* Grunow were investigated. Results permitted to devise adequate culture protocols to produce a biotechnologically important substance: an apoptogenic compound that specifically destroys the androgenic gland of a shrimp and could find novel
applications in human medicine.

3.4.3. **Feeding of Penaeus japonicus Bate (Decapoda: Penelidaea) in pond cultures: size descriptors and food selection.** (Cited by 3. I.F.= 0.83)

Various characters of food, as calorimetry, biometry, type, may influence the preferences of consumers and this may have importance also for cultured organisms. In this study weight data are coupled with the numerical abundance of prey to apply the (previously developed) index of food type and evaluate the trophic behaviour of prawn.
A Production Model for *Posidonia oceanica* Based on Temperature

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The leaf growth and plant population dynamics of the Mediterranean seagrass *Posidonia oceanica* were investigated in relation to relevant environmental factors, such as temperature and irradiance. Two stands, located at 5 and 22 m in a continuous *P. oceanica* bed off Lacco Ameno (Island of Ischia, Gulf of Naples), were studied over 1 year. The aims of the study were: (1) to investigate the influence of temperature on the spatial and temporal growth pattern of *P. oceanica*, using an *ad hoc* simulation model of the monthly plant production; and (2) to identify the most efficient descriptors of plant growth, and therefore to achieve a simple method by which shoot production could be estimated.

Irradiance is low at 22 m in the Lacco Ameno meadow, with the minimum value lower than 80 μE m$^{-2}$ s$^{-1}$ in winter. Temperature also differentiates the two water bodies in summer. The two stands differ for their structure (e.g. leaf area index), for plant production, and for some phenological features (e.g. leaf width).

The best biological descriptors of plant production were identified and an equation was derived to estimate plant growth; the equation is based on the growth of the leaf of Rank 2, which describes the growth pattern of the whole plant. Significant correlations between temperature and the production descriptors were found. A numerical model of yearly leaf growth was devised. The model was validated by predicting the growth pattern of several Mediterranean *P. oceanica* beds and comparing with observed values. It was also applied to simulate plant growth for the Lacco Ameno bed in preceding years, at both 5 and 22 m depths.

**Keywords:** growth; mathematical model; *Posidonia oceanica*; seagrasses; Gulf of Naples

*Introduction*

*Posidonia oceanica* (L.) Delile is the dominant seagrass in the Mediterranean, where it develops highly productive meadows (Wittman, 1984; Pergent & Pergent-Martini, 1991). The primary production and biomass of these meadows show defined seasonal and spatial patterns according to depth (Ott, 1980; Caye & Rossignol, 1983; Bay, 1984; Wittman, 1984; Buia et al., 1992). A bimodal pattern in the annual cycle of primary production has been described for beds shallower than 15 m, at several geographical locations (Bay, 1984; Mazzella & Ott, 1984), while a unimodal pattern, time delayed with respect to the main peak of shallow meadows, has been observed for beds deeper than 15 m (Romero, 1985; Buia et al., 1992). In addition, from year to year, maximum growth rate and biomass of *P. oceanica* are not always reached in the same months (Buia et al., 1992).

Methods currently used to evaluate seasonal plant growth are based on leaf punching and estimation of biomass production in a given time interval (Zieman, 1974; Bedhomme et al., 1983). Samples obtained by these marking methods need a laboratory treatment that is time consuming. One aim of the present paper was to identify good descriptors of leaf primary production which could allow both seasonal and spatial patterns to be followed by modification of Zieman’s (1974) and related growth estimation techniques.

Irradiance, nutrient availability and temperature are the main factors regulating primary production of marine vascular plants (Zimmerman et al., 1989, 1994; Lorenti et al., 1993; Alcoverro et al., 1995). The relationships between production, irradiance and nutrient availability have been widely investigated, but the role played by temperature on short- (seasonal) and long-term (annual) production patterns is poorly known. The main aim of the research was to assess the influence of temperature on the growth patterns of the Mediterranean *P. oceanica*. Therefore, a mathematical simulation model was developed, based on the most efficient phenological descriptors, the dynamics of appearance of new leaves, and the temporal and spatial variability of temperature.

**Methods**

*Study area*

A continuous bed of *P. oceanica* extending from 1 to 32 m off Lacco Ameno (Island of Ischia, Gulf of
Naples, Italy) was studied (Figure 1). A discontinuity in shoot density and leaf biomass exists at approximately 15 m depth, coinciding with the depth of the summer thermocline (Buia et al., 1992). The yearly production is 346 g dry weight (dw) mm$^{-2}$ year$^{-1}$ at 5 m and 101 g dw m$^{-2}$ year$^{-1}$ at 22 m depth (Buia et al., 1992). A shallow site (5 m) and a deep site (22 m) were chosen to bracket this discontinuity. In fact, the meadow at 22 m shows depth-related differences in the pattern of growth with respect to 5 m (Buia et al., 1992); a delay in reaching the maximum leaf biomass and different seasonal production patterns from those observed at 5 m were observed at the deep stand (Mazzella et al., 1989).

Data collection

Monthly samples of *P. oceanica* were collected from May 1988 to September 1989 at 5 and 22 m, i.e. above and below the observed phenological and temperature discontinuity. Each month, 20 shoots were collected from both depths for phenological studies, while an additional 20 shoots were marked according to the Zieman method (1974) and used to obtain leaf production measurements, as described in Buia et al. (1992). The length of green and brown tissue, the leaf width and the sheath's length were measured. Monthly production rates were determined. The life span of each leaf was computed by means of a life table, based on average monthly data; each leaf in the shoot was followed (rank, length, monthly production) from the date of appearance to abscission. Irradiance and water temperature were monitored monthly at both sampling stations (Figure 2 and 3). Light measurements were performed at noon, with a submersible quantameter (Biospherical mod. QS1) at subsurface level (0-1 m depth) and at the top of the leaf canopy at each station. Each value represents the mean of three replicate measurements obtained during the same day, and four time lags (1–4 months prior to the estimated production) were employed in correlation analyses to determine the scale over which light can influence growth.

Similarly, different temperature functions were employed in correlation analyses to determine the time scale over which temperature influences growth:

(1) TEM, monthly temperature;
(2) TE2, mean temperature of 2 months prior to the estimated production;
(3) TE3, temperature 3 months prior to the estimated production;
(4) TE4, temperature 4 months prior to the estimated production; and

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**Figure 1.** Location of the study site in Lacco Ameno Bay (Island of Ischia, Gulf of Naples, Italy).

**Figure 2.** Irradiance at 5 m (□) and 22 m (○) in the Lacco Ameno (Gulf of Naples) *Posidonia oceanica* bed. Each value is the average of three daily measurements obtained each month during the period of the present investigation. Error bars refer to the standard deviation on the mean of the three daily measurements.

**Figure 3.** Temperatures at 5 m (□) and 22 m (○) in the Lacco Ameno Bay. Data were collected on 1 day in each month during the study period.
Production model for *Posidonia oceanica* based on temperature

### Table 1. Main leaf features and environmental variables considered in the present study

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<tbody>
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<td>Number of intermediate leaves (no. shoot(^{-1}))</td>
<td>NNL</td>
<td>Production of new leaves (no. shoot(^{-1}) day(^{-1}))</td>
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<tr>
<td>NRA</td>
<td>Number of adult leaves (no. shoot(^{-1}))</td>
<td>SUG</td>
<td>Leaf growth (cm(^{2}) shoot(^{-1}) day(^{-1}))</td>
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<td>LEP</td>
<td>Leaf position (no.)</td>
<td>LEL</td>
<td>Leaf elongation (cm day(^{-1}))</td>
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<td>EL1</td>
<td>Elongation of Leaf 1 (cm leaf(^{-1}) day(^{-1}))</td>
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<td>LES</td>
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<td>LEA</td>
<td>Length of adult leaves (cm)</td>
<td>SG1</td>
<td>Growth of Leaf 1 (cm(^{2}) leaf(^{-1}) day(^{-1}))</td>
</tr>
<tr>
<td>LEI</td>
<td>Length of Leaf 1 (cm)</td>
<td>SG2</td>
<td>Growth of Leaf 2 (cm(^{2}) leaf(^{-1}) day(^{-1}))</td>
</tr>
<tr>
<td>LE2</td>
<td>Length of Leaf 2 (cm)</td>
<td>SG3</td>
<td>Growth of Leaf 3 (cm(^{2}) leaf(^{-1}) day(^{-1}))</td>
</tr>
<tr>
<td>LE3</td>
<td>Length of Leaf 3 (cm)</td>
<td>DSP</td>
<td>Daily shoot production (mg shoot(^{-1}) day(^{-1}))</td>
</tr>
<tr>
<td>MLE</td>
<td>Maximum leaf length (cm)</td>
<td>NRT</td>
<td>Total number of leaves (no. shoot(^{-1}))</td>
</tr>
<tr>
<td>WII</td>
<td>Width of intermediate leaves (cm)</td>
<td>AGS</td>
<td>Age of shoot (day)</td>
</tr>
<tr>
<td>WIA</td>
<td>Width of adult leaves (cm)</td>
<td>AG1</td>
<td>Age of Leaf 1 (day)</td>
</tr>
<tr>
<td>WLE</td>
<td>Width of Leaf 2 (cm)</td>
<td>AG2</td>
<td>Age of Leaf 2 (day)</td>
</tr>
<tr>
<td>BIT</td>
<td>Total shoot biomass (g dw shoot(^{-1}))</td>
<td>AG3</td>
<td>Age of Leaf 3 (day)</td>
</tr>
<tr>
<td>BEP</td>
<td>Epiphyte biomass (g dw shoot(^{-1}))</td>
<td>LLS</td>
<td>Leaf life span (months)</td>
</tr>
<tr>
<td>IRR</td>
<td>Monthly mean irradiance (µE m(^{-2}) s(^{-1}))</td>
<td>TE2</td>
<td>Mean temperature of 2 months prior to estimation of production (°C)</td>
</tr>
<tr>
<td>TEM</td>
<td>Monthly temperature (°C)</td>
<td>TE3</td>
<td>Temperature of 3 months prior to estimation of production (°C)</td>
</tr>
<tr>
<td>TED</td>
<td>Temperature variations (°C)</td>
<td>TE4</td>
<td>Temperature 4 months prior to estimation of production (°C)</td>
</tr>
</tbody>
</table>

### Data analysis

Analysis of variance (ANOVA) was performed on phenological, production and environmental data collected at 5 and 22 m, in order to contrast the two stands. The correlations between leaf and production parameters, and the abiotic factors listed in Table 1 were calculated. The relationships between biological (production of new leaves, rate of leaf elongation, several phenological parameters) and environmental (temperature and light intensity) factors were described using linear and non-linear equations. The significance of the correlation coefficients and regression analyses was tested by means of ANOVA. The best correlations obtained were applied to a simple production model to predict yearly shoot production.

### The model

A numerical model was developed to simulate shoot production. It was based on the growth of each leaf in a shoot, the monthly production of new leaves, and the effect of temperature on leaf production. It consists mainly of differential equations whose general formulation is: \(dX(t) = f(X(t))\) \(dt\), assuming that \(dX(t) = X(t+dt) - X(t)\).

The \(f\) function constants were calculated using regression analyses on temperature and production data of Lacco Ameno meadow at 5 m, according to the experimental results of the present study. In order to perform iterations on the equations for defined simulation times, the \(X(t+dt)\) computed becomes the \(X(t)\) for the next \(X(t+dt)\) at each time step, given starting values for variables and parameters. The model’s equations compute the age of each leaf taking into account its date of appearance; further calculations for elongation of each leaf are performed, taking into account its age and the water temperature, and, finally, elongation data are transformed into weight units of new tissue produced. The model was run using MS Excel software.

To validate the model, simulations of production for several Mediterranean meadows [Islas Medes, Spain; Calvi (Corsica), France; Ischia, Italy] were performed using the production and temperature data reported in the literature (Bay, 1984; Romero, 1985; Ott, 1980; Peduzzi, 1987, respectively), as well as those measured in Lacco Ameno at 22 m depth during the present study. The results obtained by simulation were compared to those obtained experimentally using a paired samples \(t\)-test on fitted vs. actual data.
A Spearman rank pairwise test was used to compare the patterns of fitted and actual data. The model was also applied to monthly temperature data measured at Ischia in 4 years preceding this study, obtaining a long-term growth simulation in the studied area.

### Results

#### Production and environmental factors

Significant differences were found between the two studied stands of the Lacco Ameno bed in both environmental (irradiance and temperature) and biological (leaf width, total number of leaves, annual production) parameters. Structural parameters of the meadow, such as shoot density and leaf area index (LAI), were also statistically different (Table 2).

Differences in irradiance between the two stations were very marked. Irradiance was low throughout the year at the deep station and in winter it fell to values close to the $I_0$ of *P. oceanica* (about 50 μE m$^{-2}$ s$^{-1}$; Lorenti et al., 1995; Figure 2). During 6 years of measurements, the mean annual trends of temperature followed the classical seasonal fluctuations observed in coastal waters of the Mediterranean Sea. The maximum values of temperature were reached with 2 months delay in the deep stand, where it never exceeded 22 °C. The water body at 5 m was subjected to temperatures higher than 20 °C for about 5 months, compared with 2 months at 22 m (Figure 3).

Some phenological parameters and features of *P. oceanica* beds showed well-defined seasonal patterns between stations. The total number of leaves per shoot varied between the two stands of *P. oceanica*. In particular, at 5 m, the maximum number of leaves was reached in November (10 leaves) and the minimum number was reached in May (seven leaves); at 22 m, the maximum number occurred in both November and April (eight leaves; Figure 4[a]). The monthly rate of leaf appearance also showed defined patterns in the two stands, with higher rates in autumn–winter (October–January) and lower rates in spring–summer [March–July; Figure 4(b)]. The linear relationship between leaf appearance and the temperature of 4 months prior to the measurement (NNL= 0.17 $\times$ TE4 – 2.35; Table 3), shows that the new leaf production was equal to zero at both depths under 14 °C. Leaf fall was almost constant during the year at 5 m, while a seasonal trend could be identified at 22 m. The number of leaves produced yearly were nine at 5 m and seven at 22 m.

The growth pattern of single leaves varied according to the month in which they appeared in the shoot [Figure 5(a,b)]. The elongation of single leaves at 5 m was lowest for leaves appearing in June and growing in summer; it was highest for leaves appearing in September and growing in autumn–winter [Figure 5(a)]. A similar pattern was found at 22 m, with a

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**Table 2.** Variance analysis (ANOVA) performed on phenological, production and environmental parameters at 5 and 22 m stations. Sets of 14 monthly data were compared

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean 5 m</th>
<th>Mean 22 m</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAI</td>
<td>6.18</td>
<td>2.43</td>
<td>39.75</td>
<td>0.000</td>
</tr>
<tr>
<td>DEN</td>
<td>341.00</td>
<td>163.00</td>
<td>55.00</td>
<td>0.000</td>
</tr>
<tr>
<td>DSP</td>
<td>3.06</td>
<td>2.06</td>
<td>5.42</td>
<td>0.027</td>
</tr>
<tr>
<td>SUG</td>
<td>0.99</td>
<td>0.66</td>
<td>4.23</td>
<td>0.049</td>
</tr>
<tr>
<td>EL2</td>
<td>0.34</td>
<td>0.28</td>
<td>1.84</td>
<td>0.186</td>
</tr>
<tr>
<td>NNL</td>
<td>0.78</td>
<td>0.60</td>
<td>0.57</td>
<td>0.466</td>
</tr>
<tr>
<td>NRT</td>
<td>8.09</td>
<td>7.56</td>
<td>4.10</td>
<td>0.053</td>
</tr>
<tr>
<td>NRI</td>
<td>1.65</td>
<td>1.43</td>
<td>0.43</td>
<td>0.520</td>
</tr>
<tr>
<td>NRA</td>
<td>3.06</td>
<td>2.89</td>
<td>1.24</td>
<td>0.276</td>
</tr>
<tr>
<td>WII</td>
<td>0.99</td>
<td>0.89</td>
<td>13.72</td>
<td>0.001</td>
</tr>
<tr>
<td>WIA</td>
<td>1.03</td>
<td>0.92</td>
<td>9.75</td>
<td>0.004</td>
</tr>
<tr>
<td>WI2</td>
<td>0.99</td>
<td>0.88</td>
<td>32.03</td>
<td>0.000</td>
</tr>
<tr>
<td>LEI</td>
<td>28.00</td>
<td>28.34</td>
<td>0.00</td>
<td>0.959</td>
</tr>
<tr>
<td>LEA</td>
<td>46.22</td>
<td>45.03</td>
<td>0.04</td>
<td>0.837</td>
</tr>
<tr>
<td>TEM</td>
<td>20.32</td>
<td>17.30</td>
<td>4.52</td>
<td>0.042</td>
</tr>
<tr>
<td>IRR</td>
<td>522.86</td>
<td>127.07</td>
<td>126.86</td>
<td>0.000</td>
</tr>
</tbody>
</table>

See Table 1 for definitions of abbreviation codes.
delay of about 2 months in the appearance of the leaf which reaches the maximum elongation rate [Figure 5(b)]. A correlation was found between the sheath length and the position of the leaf in the shoot, both at 5 and 22 m (LES/LEP; Table 3). Moreover, at 5 m, there was a significant inverse correlation between the leaf life span (months) and the mean temperature over the leaf life span (LLS/TLS; Table 3). The maximum life span was about 10 months at both stands. At 5 m, longer life span of leaves appearing in winter was accompanied by a longer length; leaves appearing in summer had a shorter life span and a shorter length [Figure 6(a)]. These differences were less marked at 22 m; however, leaves appearing in June and September and growing in winter at 22 m were shorter [Figure 6(b)].

Leaves of different ages and ranks had different growth rates. In particular, leaves of Ranks 5–7 showed the lowest rate of elongation at both stands (Figure 7(a,b)). Leaves 1–4 had a higher elongation rate and a bimodal trend with maxima in October and May at 5 m [Figure 7(a)]. At 22 m, the maximum growth was shown by Leaves 1–3 in May [Figure 7(b)]. At both stations, significant correlation was found between the daily shoot production and the elongation of Leaf 2 (DSP/EL2; Table 3).

Interestingly, the daily shoot production (DSP, as mg dw shoot$^{-1}$ day$^{-1}$) was related to the elongation of the second leaf (EL2, as cm day$^{-1}$) at both depths, according to the following equations:

\[
\text{DSP} = 8.88 \times \text{EL2} + 0.08 \quad (\text{at } 5 \text{ m}; \ r=0.79)
\]

\[
\text{DSP} = 6.34 \times \text{EL2} + 0.31 \quad (\text{at } 22 \text{ m}; \ r=0.89)
\]

The daily shoot production at both stations was related (Table 3) to the length of intermediate leaves (DSP/LEI) and the elongation of Leaf 2 (DSP/EL2), which in its turn was related to the number of intermediate leaves (EL2/NRI). The daily shoot production was also correlated to the number of intermediate leaves (DSP/NRI) and the width of adult leaves (DSP/WIA) at 5 m. Shoot production followed the seasonal trend of leaf width at 5 m (DSP/WIA; \(r=0.81\)). Variations of temperature at both 5 and 22 m were positively related to the age of Leaf 2 (TED/AG2). At 22 m, irradiance was positively related to the length of adult leaves (IRR/LEA), to the shoot biomass (IRR/BIT) and to the age of Leaf 2 (IRR/AG2; Table 3). At 5 m, this parameter was negatively related to total number of leaves (IRR/NRT) and growth (IRR/SUG), and positively related to the age of Leaf 2.

### Table 3. Correlation coefficients ($r$) and probability levels ($P$) computed between the most efficient phenological and production descriptors, and environmental variables, for the 5 and 22 m stands off Lacco Ameno (Ischia, Gulf of Naples)

<table>
<thead>
<tr>
<th></th>
<th>5 m $r$</th>
<th>5 m $P$</th>
<th>22 m $r$</th>
<th>22 m $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSP/EL1</td>
<td>0.49</td>
<td>0.000</td>
<td>0.65</td>
<td>0.000</td>
</tr>
<tr>
<td>DSP/EL2</td>
<td>0.79</td>
<td>0.000</td>
<td>0.89</td>
<td>0.000</td>
</tr>
<tr>
<td>DSP/EL3</td>
<td>0.83</td>
<td>0.000</td>
<td>0.68</td>
<td>0.000</td>
</tr>
<tr>
<td>DSP/LEI</td>
<td>0.47</td>
<td>0.019</td>
<td>0.66</td>
<td>0.009</td>
</tr>
<tr>
<td>DSP/NRI</td>
<td>0.81</td>
<td>0.000</td>
<td>0.45</td>
<td>0.102</td>
</tr>
<tr>
<td>DSP/WIA</td>
<td>0.81</td>
<td>0.000</td>
<td>0.01</td>
<td>0.977</td>
</tr>
<tr>
<td>EL2/LEI</td>
<td>0.31</td>
<td>0.288</td>
<td>0.78</td>
<td>0.001</td>
</tr>
<tr>
<td>EL2/NRI</td>
<td>0.71</td>
<td>0.005</td>
<td>0.72</td>
<td>0.003</td>
</tr>
<tr>
<td>IRR/AG2</td>
<td>0.65</td>
<td>0.010</td>
<td>0.80</td>
<td>0.000</td>
</tr>
<tr>
<td>IRR/BIT</td>
<td>0.24</td>
<td>0.406</td>
<td>0.73</td>
<td>0.002</td>
</tr>
<tr>
<td>IRR/LEA</td>
<td>0.47</td>
<td>0.080</td>
<td>0.70</td>
<td>0.005</td>
</tr>
<tr>
<td>IRR/NRT</td>
<td>0.70</td>
<td>0.011</td>
<td>0.42</td>
<td>0.137</td>
</tr>
<tr>
<td>IRR/SUG</td>
<td>0.72</td>
<td>0.003</td>
<td>0.05</td>
<td>0.868</td>
</tr>
<tr>
<td>LES/LEI</td>
<td>0.81</td>
<td>0.000</td>
<td>0.87</td>
<td>0.000</td>
</tr>
<tr>
<td>LES/LEP</td>
<td>0.78</td>
<td>0.000</td>
<td>0.85</td>
<td>0.000</td>
</tr>
<tr>
<td>LLS/TLS</td>
<td>0.83</td>
<td>0.011</td>
<td>0.53</td>
<td>0.210</td>
</tr>
<tr>
<td>MLE/LLS</td>
<td>0.78</td>
<td>0.000</td>
<td>0.37</td>
<td>0.000</td>
</tr>
<tr>
<td>NNL/TE4</td>
<td>0.69</td>
<td>0.009</td>
<td>0.65</td>
<td>0.015</td>
</tr>
<tr>
<td>TED/AG2</td>
<td>0.72</td>
<td>0.0005</td>
<td>0.77</td>
<td>0.002</td>
</tr>
<tr>
<td>TED/DSP</td>
<td>0.31</td>
<td>0.300</td>
<td>0.27</td>
<td>0.360</td>
</tr>
<tr>
<td>TED/EL2</td>
<td>0.48</td>
<td>0.099</td>
<td>0.15</td>
<td>0.614</td>
</tr>
</tbody>
</table>

See Table 1 for definitions of abbreviation codes.
Temperature and irradiance, at both 5 and 22 m, were scarcely correlated ($r=0.4$ and $0.3$, respectively).

The model

Number of new leaves computation. Posidonia production was computed over a 1-year period using a 1-day time step, while a check for the number of new leaves that had appeared (NNL) was simulated using a time step of 30 days. As it was demonstrated that the appearance of new leaves was best related to the temperature 4 months prior to leaf production, NNL was calculated using Equation 3, which was obtained by regression of experimental data collected at Lacco Ameno at 5 m depth. The results were rounded to the nearest integer.

$$\text{NNL} = (0.17 \times \text{TE4}) - 2.35 \quad (3)$$

From Equation 3, it is possible to obtain two, one or zero leaves monthly (NNL), depending on the temperature measured 4 months prior to calculation time (TE4).

Based on the appearance of a new leaf in the shoot, a new arrangement was computed for leaf positions and relative leaf age. New leaves assumed the first (and the second if two) position(s) and age(s), making the others shift up one place. As the results demonstrated that the production of leaves of Rank 7 or higher is negligible, only the first seven leaves were taken into account in the model. Any older leaves in the shoot (Rank 8 or more) are considered to be shed.

Age of leaf computation. The function Age, defines the age of each leaf in a shoot, from rank $i=1$ to 7. Equation 4 is used to calculate the age of each leaf in a shoot, according to its rank:

$$\text{Age}_{i(t)} = \text{Age}_{i(t-d_t)} + (d_t + \text{Shift}_{i-1} - \text{Shift}_i) \quad (4)$$

where $i$ is the rank of the leaf and $t$ is the time. The forcing function Shift, rearranges the age of each leaf, taking into account the shift to higher ranks due to the appearance of a new leaf. The values assumed by this function, therefore, depend on the number of new leaves appearing (taking into account a maximum of two leaves per month), as follows:

\[
\begin{align*}
\text{Shift}_t &= 0 & \text{NNL} &= 0 \\
\text{Shift}_t &= \text{Age}_t & \text{for NNL} &= 1 \\
\text{Shift}_t &= \text{Age}_t + \text{Age} - 1 & \text{for NNL} &= 2
\end{align*}
\]
Daily leaf elongation computation. Daily leaf elongation (cm day$^{-1}$) was calculated using equations, proper for any leaf, modified from Draper and Smith (1981), whose parameters were obtained experimentally, taking into account the data collected in Lacco Ameno at 5 m depth:

$$EL_i = \frac{e^{(3.85 - Age_i)^{0.64}}}{(3.85 - Age_i)^2 + 3.03}$$

(5)

Shoot production computation. Daily elongation of each leaf (EL$_i$, expressed as cm day$^{-1}$) was transformed into daily production of new tissue (DLP$_i$, expressed as mg dw day$^{-1}$) using Equation 6 which was obtained from a linear relation, using the data collected in Lacco Ameno at 5 m depth:

$$DLP_i = 2.48 \times EL_i(t) + 0.499$$

(6)

The daily shoot production was computed by the function DSP, as follows:

$$DSP = \sum_{i=1}^{n} DLP_i$$

(7)

The monthly shoot production was then calculated to plot the yearly production pattern.

Simulations

Validation tests indicated no significant differences between fitted and observed data in Lacco Ameno, Ischia, at 22 m (data collected during the present investigation; Figure 8), and Islas Medes, Spain, at 5 m [Romero, 1985; Figure 9(a)]. Simulations performed on data by Ott (1980) and Bay (1984) produced significant differences between fitted and observed data [Figure 9(b,c)]. However, the simulated yearly production had the same trends and slopes as the actual data, as in previous tests. In fact, Spearman tests indicated a $\rho=0.94$ between fitted and actual data by Bay (1984), and a $\rho=0.82$ for the data by Ott (1980). Regression analysis between observed and fitted production data indicated that the differences are constant for a given site, and they can be described by the slope of the regression, $K$, which assumes values of 1, 2.23 and 0.81 for Lacco Ameno, Castello and Calvi, respectively. Therefore, Equation 5 can be modified including a calibration factor, $K$, to be used for different environmental conditions.
Grass populations (McMillan & Phillips, 1979) have been found to be a good descriptor for other seagrass populations. Leaf width has been identified as the best descriptor at plant level, while shoot density and LAI were the best characterizing the populations of 5 and 22 m depth. Leaf width and leaf life span were the biological factors that showed a strong relationship with leaf growth and temperature. In the present investigation, leaf growth was found to be influenced by temperature data recorded from 1984 to 1987 at each depth.

The seasonal variability of temperature shows a strong relationship between leaf growth and temperature. In the Lacco Ameno meadow has been revealed by other investigations (Buia et al., 1993, 1995). The role of temperature on growth has been demonstrated for other seagrasses (Fong & Harwell, 1994; Walker & Cambridge, 1995) and for Potamogotonaceae (Spencer & Ksander, 1992), and the present investigation confirms the importance of temperature for P. oceanica in the Mediterranean (Buia & Mazzella, 1991).

The seasonal variability of temperature shows a time scale close to that of the plant production at both the shallow and deep stands. Therefore, the authors were able to obtain a predictive model based on temperature alone, by linking this factor to the most efficient biological descriptors of growth (i.e. leaf elongation and age). The model accurately simulated the production data obtained experimentally for 22 m depth. Elongation is similarly highest in the youngest (second and third) leaves in Zostera marina (Sand-Jensen, 1975; Mukai et al., 1979; Kemp et al., 1987). The finding of leaves representative of the whole shoot growth in P. oceanica can lead to new effortless methods for the estimation of seagrass production (Zieman & Wetzel, 1980).

The efficacy of a few descriptors of shoot growth (such as elongation of Leaf 2, or the growth of the youngest leaves) could help to reduce the effort for laboratory process of samples marked by the Zieman method (1974).

Noteworthy are the different seasonal patterns between shallow and deep stand. The leaf life span, which in this phanerogam is very long compared to other species (such as Cymodocea nodosa, unpubl. data; Z. marina, Jacobs, 1979; Zostera muellleri, Kerr & Strother, 1989; Heterozostera tasmanica, Bulthuis & Woelkeling, 1983; Duarte, 1991a), is longer in the deep stand than in the shallow stand. This derives from lower growth rate of leaves, and possibly also from lower hydrodynamic forces at higher depth, which reduces leaf shedding (Gambi et al., 1989). The leaf assemblage of the shoot (number of leaves, age of each leaf, etc.) can influence the growth rhythm for a long period, and represents a ‘memory’ for the growth mechanism. The lowest growth rate and the shortest life span were observed in summer at 5 m, related to a high temperature. In contrast, at 22 m, the lowest growth rate was accompanied by a longer life span in autumn–winter, when the lowest in situ irradiance was recorded. Therefore, environmental factors influence P. oceanica leaf growth dynamics, although internal mechanisms regulate growth as demonstrated in previous work (Ott, 1979).

The influence of light on growth in the Lacco Ameno meadow has been revealed by other investigations; irradiance was found to be limiting only for the deep stand (Buia et al., 1992; Lorenti et al., 1993, 1995). The role of temperature on growth has been demonstrated for other seagrasses (Fong & Harwell, 1994; Walker & Cambridge, 1995) and for Potamogotonaceae (Spencer & Ksander, 1992), and the present investigation confirms the importance of temperature for P. oceanica in the Mediterranean (Buia & Mazzella, 1991).

The seasonal variability of temperature shows a time scale close to that of the plant production at both the shallow and deep stands. Therefore, the authors were able to obtain a predictive model based on temperature alone, by linking this factor to the most efficient biological descriptors of growth (i.e. leaf elongation and age). The model accurately simulated the production data obtained experimentally for 22 m depth.

\[ EL = \frac{(e^{(3.85 - \text{Age}_i)^3(\text{TE}-1)^7)-0.064})}{(3.85 - \text{Age}_i)^2 + 3.03} \times K \]
in Lacco Ameno during the present investigation, and the data reported by Romero (1985) for a meadow distant from the present study site. Small differences between actual and fitted data are lower than the differences observed among results obtained by different methods of measurement (Romero, 1985).

The differences between simulated and observed data in Castello and Calvi at 5m depth can be attributed to the influence of other environmental factors (e.g. nutrient availability, irradiance; Alcoverro et al., 1995; Lorenti et al., 1995) not considered in the present model. The simulation demonstrated that the actual data collected by Ott (1980) at Castello are higher than the fitted data but show the same trend, as confirmed by the Spearman rank test. Data fitted for the meadow at Calvi are slightly higher than the actual data collected by Bay (1984), although the seasonal patterns are perfectly reproduced ($\rho=0.94$).

Light and nutrients represent the input of energy and matter for growth, and influence yearly productivity (Delgado, 1986; Dennison et al., 1987; Lorenti et al., 1995). The present results indicate that temperature represents a modulating factor, discriminating different bodies of water and influencing the seasonal growth pattern. Therefore, the total yearly production yield is mainly influenced by light and nutrients (Pirc, 1985a,b; Williams & Ruckelshaus, 1993), while the seasonal production pattern is modulated by temperature.

Experimental data suggest that a variety of physical and biological factors are acting in a potential hierarchical fashion to determine leaf elongation. Responses of $P$. oceanica to the temperature 3–4 months prior to production appear consistent with the assumption that $P$. oceanica has a very slow metabolism and the capability to store carbohydrate reserves in the rhizomes in spring, to support winter leaf elongation (Pirc, 1985b, 1989). Small differences in monthly production, obtained when simulating the growth pattern of the stand at 22m depth, may be attributable to long-term adaptations of the plant to a different thermal regime and to the effect of limiting factors (e.g. irradiance). Further research applying the model herein obtained to gradients of environmental factors, besides the temperature, will allow a standarization of the factor $K$ and a comparison of environmental factors acting on the growth processes. The similarity between actual production data and fitted data obtained by the present model (based on a data set collected at 5m in Lacco Ameno), when applied to various depths in several geographical regions, confirms that temperature represents the main factor regulating the seasonal variability of growth. This finding is in accordance with the results obtained for $Thalassia testudinum$ (Dawes et al., 1986; Tomasko & Dawe, 1990; Fong & Harwell, 1994). In fact, seasonal trends in productivity and biomass of $T$. testudinum have been attributed to temperature variations. Moreover, the growth patterns obtained when applying the present model to 5 and 22m depths (presence of a time delay in the production at 22m, lower production in deep stand, etc.) indicate that temperature also has an effect on the separation of the two stands.

The significant differences obtained in the simulated production patterns of the two stands at Lacco Ameno indicate that temperature, as well as irradiance (Lorenti et al., 1995), can regulate growth processes even at a small spatial and temporal scale. Irradiance shows a higher variability in space and time, and it probably controls the maximum depth penetration of $P$. oceanica (Chambers & Kalff, 1985; Duarte, 1991b; Pirc, 1984). Temperature can be considered a conservative parameter to which $P$. oceanica responds over long time periods and which overlaps the influence of light.

Acknowledgements

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CULTURE CONDITIONS INFLUENCE THE GROWTH DYNAMICS AND THE PRODUCTION OF COCCONEIS SCUTELLUM (BACILLARIOPHYTA)\(^1\)

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The optimal conditions for the growth of two con-specific benthic diatoms were defined through factorial experimentation. We investigated the roles of light spectrum, nutrient availability, and culture conditions on the laboratory production of Cocconeis scutellum Ehrenb. and C. scutellum parva Grunow. Diatoms were cultivated in petri dishes, and inverted optical microscopy was used to periodically record their abundance. Growth curves were constructed from these data for each culture condition. In addition, at the end of the experiment we performed weight measurements to determine the total production for each of the considered conditions. We found that cultivation in nonsealed (NS) petri dishes (permitting gas exchange) represented the most productive technique. Cell density and biomass varied among light spectra, although this effect was inconsistent. For example, the Sylvania Gro-Lux lamp (GL) produced the lowest cell density but highest biomass, suggesting that it may promote the production of larger cells. Surprisingly, of the culture media tested, f/2 (a media commonly used for the culture of diatoms) was the least productive. Diatom density and biomass were variably dependent on the combination of experimental culture conditions and strain used. These physical and chemical factors act mainly on given features of the diatom growth curve. These results permitted us to devise adequate culture protocols, to produce a biotechnologically important substance: a proapoptotic compound that specifically destroys the androgenic gland of a shrimp and could find novel applications in human medicine.

**Key index words:** apoptosis; Cocconeis; cultivation; gas; growth; light spectrum; medium; metabolite; strain

**Abbreviations:** Aq, Philips Aquarelle lamp; AS, Sylvania Aquastar lamp; C.A., correspondence analysis; C1, Cocconeis scutellum var. scutellum; C2, Cocconeis scutellum var. parva; dwt, dry weight; f/2, Guillard’s f/2 culture medium; GL, Sylvania Gro-Lux lamp; GR, growth rate; K, Keller culture medium; MJ, modified Jørgensen culture medium; NS, nonsealed; S, sealed

Among the eukaryotic phytoplankton, diatoms are responsible for \(\sim 40\%\) of the marine primary productivity (Falkowski et al. 1998). Despite their abundance and diversity in nature (Norton et al. 1996, van den Hoek et al. 1997), both in the plankton and in the benthos, few species are cultivated in aquaculture plants or for relevant biotechnology products (Borowitzka 1994). Microalgae and, in particular, diatoms are known to produce very large, not yet completely elucidated sets of secondary metabolites (Lincoln et al. 1990, Nappo et al. 2009). While there is a good understanding of cultivation techniques to be applied to both planktonic and benthic diatoms, the effects and interactions of selected environmental conditions on the population dynamics and the productive yields of cultivated diatoms are yet to be fully understood (de la Peña 2007). Only in the past decade have experiments aimed at understanding the effect of light (Goksan et al. 2003), scale (Lebeau and Robert 2003a), nutrient limitation (Davidson and Gurney 1999), and other environmental conditions (Marchetti et al. 2004) indicated that productivity and growth dynamics of diatoms are dramatically influenced by the culture procedure. In addition, several articles showed that the physiological status of diatoms varies according to the presence/absence of some nutrients (Eker-Devely et al. 2006, Chundi et al. 2007), presence of pollutants (Kawakami et al. 2006), quality of light spectrum (Mouget et al. 2005), irradiance (Ranieri et al. 2007), and so forth. All these conditions may influence the production of secondary metabolites according to the culture conditions (Popp et al. 2006, Affan et al. 2007).

In fact, one aim for cultivating diatoms may be the production of secondary metabolites, which are used for various biotechnological purposes (Lebeau and Robert 2003b) or for the nutrition of aquaculture relevant organisms (de la Peña 2007). For example, benthic diatoms are important inducers of larval settlement of abalones and food for their early juvenile stages (Uki and Kikuchi 1979, Ebert and Houk 1984, Kawamura et al. 1995). In addition, only a few studies addressed the effect of multiple factors, as the colimitation of growth due to phosphorus and light (Hill and Fanta 2008) and the interactions of various nutrients (Davidson and Gurney 1999), which make this matter very complex and, sometimes, lead to unpredictable results (Affan et al. 2007).

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This situation is complicated by the effect of strains (Lakeman et al. 2009), since the production efficiency of diatom secondary metabolites often depends on the strain more than on the species (Liang et al. 2005, Thessen et al. 2009). The genetic variability of diatoms may change during time, when they are replicated in culture, along with their ability to produce selected active compounds, and it was argued that evolutionary adaptations exist and nonadaptive changes are induced by the culture conditions under which the algae are maintained (Elena and Lenski 2003). Long-term physiological changes are likely to occur when a strain is held in the laboratory for several years (Thessen et al. 2009). For example, *Pseudo-nitzschia* strains typically lose toxicity the longer they are grown in the laboratory, possibly due to interactions with bacteria (Stewart 2008), which can change as the strain ages (Kaczmarzka et al. 2005).

Therefore, to obtain accurate predictions about the ability of selected diatoms to produce bioactive compounds, we need to model the growth based on the multiple relationships existing between light intensity and spectrum, nutrient availability in the culture medium, general culture conditions, and strain features (Bates et al. 1991). The problem is even more complex when the culture of benthic diatoms is explored, since techniques for planktonic algae are traditionally devised (Couteau 1996), while the massive cultivation of diatoms growing tightly attached to a solid substrate, often in monolayer thin films, implies several additional constraints (Roberts et al. 2000).

To investigate this matter and to evaluate the importance of individual factors in the productivity and bioactivity of diatoms, we cultivated two subspecies of *Cocconeis*, namely, *C. scutellum scutellum* and *C. scutellum parva*, in the laboratory under three different fluorescent lamps (which vary in light spectrum), in two culture conditions (sealed [S] and NS petri dishes), in three culture media. We analyzed the results obtained (numerical growth curves and weight gain) to evaluate the multiple effects in a factorial experiment (Graham et al. 2005).

This diatom was chosen because it produces well-known effects on the shrimp *Hippolyte inermis*. Several species of *Cocconeis* are recognized to induce the sex reversal of this shrimp, in the field (Zupo 2001), due to the early apoptosis of the androgenic gland and of the male gonad (Zupo and Messina 2007). The active compound could find applications in human medicine, for the selective destruction of cancer cells (Zupo et al. 2007, Nappo et al. 2009). For this reason, various strains of several species of diatoms have been cultivated for years, observing variable levels of apoptogenic activity detected by the shift of the shrimp sex ratio (higher percentage of females) when cultured diatoms were added to their diet (Zupo 2000). The present investigation aimed at finding culture conditions able to maximize both the production in terms of dry weight (dwt; which is very low for benthic diatoms, in culture; Raniello et al. 2007) and the synthesis of the active metabolites (Wen and Chen 2000). The results may shed light on the factors (Acién Fernández et al. 2000) reciprocally influencing the production of bioactive compounds in cultured diatoms.

**MATERIALS AND METHODS**

*Stock cultures.* Strains of *C. scutellum var. scutellum* (C1) and *C. scutellum var. parva* (C2) were used in this experiment. These diatoms are routinely cultured at the Benthic Ecology Laboratory of the Stazione Zoologica Anton Dohrn, in Ischia. The strains were collected in the Bay of Naples, Italy (40.44.7 N, 13.56.4 E) in April 2001, isolated by a Leica micromanipulator, cleaned of bacteria and other diatoms through several passages in sterilized seawater, then identified under the SEM microscope (Jüttermann et al. 2010). Stock cultures are held in plastic multiwell plates and transferred biweekly into new sterile medium (Guillard’s f/2, Sigma Aldrich Ltd., St. Louis, MO, USA; Guillard and Ryther 1962) using autoclaved Pasteur pipettes. A sample of each strain was collected from the stock cultures and multiplied in glass petri dishes prior to starting the experiments. All the experiments described have been conducted consistently using samples of the diatoms reported above, since our aim was not to detect the well-known intraspecific diversity (Wood and Leatham 1992, Thessen et al. 2009), but the effect of variable culture conditions on the production and metabolism of two closely related varieties of *C. scutellum*. This approach has been recommended by Murphy (1978) for comparative physiological studies.

*Experimental plan.* Our aim was to devise an adequate culture method, able to maximize both the production in terms of dwt and the synthesis of the active metabolite (a proapoptotic compound to be tested on shrimp’s gonads). For this reason, the experiments reported herein were preceded by the testing of various photobioreactors (based on glass beads contained in sterile tubing and large surface reactors provided with a wave generator). These devices demonstrated acceptable production levels (not discussed herein; see Raniello et al. 2007), but very scarce production of the bioactive compound (Nappo et al. 2009). Hence, we reconsidered the classical method of production based on petri dishes, which appeared reliable and proved to produce sufficient amounts of the proapoptotic compound. Factorial experiments were designed to establish the culture conditions able to maximize biomass production, while conserving sufficient amounts of the active compound.

For this reason, four factors were considered:

1. Diatom variety: we tested two *C. scutellum* varieties, which share similar needs, coexist in the *Posidonia oceanica* leaf environment (De Stefano et al. 2008), and are both known to produce the active compound (Zupo et al. 2007), to determine whether or not they reacted in the same way to variations in selected physical and chemical factors.

2. Light spectrum: we tested the effect of three neon lamps, all producing high proportions of blue and red radiations (phytostimulant lamps) but with slight differences in the spectrum produced, to check whether or not minor variations of the light spectrum could influence diatom growth and productivity (Mouget et al. 2005). The light irradiance was maintained at a constant level, by modifying the number and position of the lamps, to reach a total irradiance of 90 μE·m⁻²·s⁻². This irradiance has been demonstrated to be adequate for the cultivation of *Cocconeis* (Raniello et al. 2007). The tested lamps were Philips (Philips S.p.A., Monza,
FACTORS CONDITIONING DIATOM PRODUCTION

Table 1. Composition of the three media tested: f/2 according to Guillard (1975), K according to Keller and Guillard (1985), and MJ according to Barsanti and Gualtieri (2006).

<table>
<thead>
<tr>
<th>Component</th>
<th>f/2</th>
<th>K</th>
<th>MJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoCl₂ · 6 H₂O</td>
<td>4.20 × 10⁻⁸</td>
<td>5.00 × 10⁻⁸</td>
<td>–</td>
</tr>
<tr>
<td>CuSO₄ · 5 H₂O</td>
<td>3.93 × 10⁻⁸</td>
<td>1.00 × 10⁻⁸</td>
<td>1.15 × 10⁻⁷</td>
</tr>
<tr>
<td>FeCl₃ · 6 H₂O</td>
<td>1.17 × 10⁻⁵</td>
<td>1.17 × 10⁻⁵</td>
<td>–</td>
</tr>
<tr>
<td>FeSO₄ · 7 H₂O</td>
<td>–</td>
<td>–</td>
<td>1.97 × 10⁻⁵</td>
</tr>
<tr>
<td>H₂SeO₃</td>
<td>–</td>
<td>–</td>
<td>1.00 × 10⁻⁸</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>–</td>
<td>–</td>
<td>2.42 × 10⁻⁵</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>–</td>
<td>–</td>
<td>6.0 × 10⁻³</td>
</tr>
<tr>
<td>MnCl₂ · 4 H₂O</td>
<td>9.10 × 10⁻⁷</td>
<td>9.00 × 10⁻⁷</td>
<td>7.94 × 10⁻⁶</td>
</tr>
<tr>
<td>Na₂ b-glycerophosphate · 6 H₂O</td>
<td>–</td>
<td>1.00 × 10⁻⁵</td>
<td>–</td>
</tr>
<tr>
<td>Na₂EDTA · 2 H₂O</td>
<td>1.17 × 10⁻⁵</td>
<td>1.11 × 10⁻⁴</td>
<td>3.0 × 10⁻²</td>
</tr>
<tr>
<td>(NH₄)₁₂M₃O₄</td>
<td>–</td>
<td>–</td>
<td>1.84 × 10⁻⁸</td>
</tr>
<tr>
<td>Na₂MoO₄ · 2H₂O</td>
<td>2.60 × 10⁻⁸</td>
<td>2.60 × 10⁻⁸</td>
<td>–</td>
</tr>
<tr>
<td>Na₂SiO₃ · 9 H₂O</td>
<td>1.06 × 10⁻⁴</td>
<td>5.04 × 10⁻⁴</td>
<td>8.0 × 10⁻⁴</td>
</tr>
<tr>
<td>NaH₂PO₄ H₂O</td>
<td>3.62 × 10⁻⁵</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>8.82 × 10⁻⁴</td>
<td>8.82 × 10⁻⁴</td>
<td>4.0 × 10⁻³</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>–</td>
<td>5.00 × 10⁻⁵</td>
<td>–</td>
</tr>
<tr>
<td>NH₄VO₃</td>
<td>–</td>
<td>–</td>
<td>2.13 × 10⁻⁷</td>
</tr>
<tr>
<td>ZnSO₄ · 7 H₂O</td>
<td>7.65 × 10⁻⁸</td>
<td>8.00 × 10⁻⁸</td>
<td>1.54 × 10⁻⁷</td>
</tr>
<tr>
<td>Tris-base (pH 7.2)</td>
<td>–</td>
<td>1.00 × 10⁻⁵</td>
<td>–</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>–</td>
<td>–</td>
<td>1.56 × 10⁻⁵</td>
</tr>
<tr>
<td>Thiamine HCl (vit. B1)</td>
<td>2.96 × 10⁻⁷</td>
<td>2.96 × 10⁻⁷</td>
<td>2.96 × 10⁻⁷</td>
</tr>
<tr>
<td>Biotin (vit. H)</td>
<td>2.05 × 10⁻⁹</td>
<td>2.05 × 10⁻⁹</td>
<td>2.05 × 10⁻⁹</td>
</tr>
<tr>
<td>Cyanocobalamin (vit. B12)</td>
<td>3.69 × 10⁻¹⁰</td>
<td>3.69 × 10⁻¹⁰</td>
<td>3.69 × 10⁻¹⁰</td>
</tr>
</tbody>
</table>

f/2, Guillard’s f/2 culture medium; K, Keller culture medium; MJ, modified Jørgensen culture medium.

3. Culture conditions, by considering two different methods:
   - petri dishes sealed by means of Parafilm® sheets (Bemis Inc., Neenah, WI, USA) or unsealed dishes. Some authors (e.g., Raniello et al. 2007) suggest that the use of this film is important in limiting evaporation during culture and reducing the introduction of bacteria. Others (e.g., Gordon et al. 2004) have not used such sealing to maximize gas exchange. We aimed at testing whether the stress due to gas exchange limitation or, alternatively, a slight change in salinity due to evaporation, could influence the diatom productivity, in relation to the other factors tested.

4. Culture media (Table 1): our mother cultures have successfully been transferred for 7 years in Guillard’s f/2, and this medium was widely used by various authors (e.g., Affan et al. 2007) for the mass culture of diatoms. It is also available in commercial premixed form. However, other culture media could increase the productivity. We tested the effect of the modified Jørgensen culture medium (MJ; Barsanti and Gualtieri 2006) and of Keller culture medium (K; Keller and Guillard 1985) in comparison with Guillard’s f/2 culture medium (f/2; Guillard 1975) on the growth of C. scutellum parva, in NS conditions, when exposed to GL light. The three media exhibit a similar elemental composition. However, some compounds are specific of individual recipes—for example, K₂HPO₄ and citric acid are present only in MJ; NaH₂PO₄ is present only in f/2; Na₂ b-glycerophosphate, H₂SeO₃, and Tris-base are present only in the K medium (Table 1). Some other compounds exhibit striking differences in abundance, as Na₂SiO₃, which is present in the MJ medium at a concentration about eight times higher than in f/2.

These four factors were considered for drawing our experimental plan (Table 2), to identify optimal conditions for promoting the productivity of Cocconeis spp. Significance of differences among treatments was evaluated by means of analysis of variance (ANOVA; performed by Statistica version 6.0, StatSoft Italia, Vigonzola, Padova, Italy), after determination of homogeneity of variance.

Diatom cultures. Besides defined experimental factors (strain, light spectrum, medium, sealing), each culture was conducted in identical culture conditions in a thermostatic chamber at 18°C, 12:12 light:dark (LD) photoperiod, and irradiance of ~90 μmol m⁻² s⁻¹, in glass petri dishes with a
diameter of 14 cm, each containing 100 mL of sterile culture media. All operations were performed under a laminar flow hood, using autoclaved materials.

Prior to starting the experiment, the diatoms were sampled from mother cultures (contained in sterile plastic multiwell plates) using a Pasteur pipette and initially transferred in 7 cm glass petri dishes containing 30 mL of culture media. These cultures were incubated for 14 d in a thermostatic chamber and then checked for the absence of bacteria and other contaminants. The growth of cells in each dish was also monitored. The diatom films grown were scraped by means of a sterile Pasteur pipette and transferred into a sterile beaker. Cell concentration was determined by means of a Neubauer counting chamber (EMS Inc., Hatfield, PA, USA), and 45 petri dishes (7 cm) were inoculated with this suspension and incubated for 14 d in a thermostatic chamber. The previous check and collection procedure was then repeated on these petri dishes and the sterile cell suspension obtained was used to start the experiments.

Numerical growth experiments. Twenty replicate petri dishes were inoculated for each of the eight treatments (according to light, strain, and “S” vs. “NS” conditions; see Table 2a), giving a total of 160 individual plates. For the second part, 20 petri dishes were inoculated for each culture medium treatment (see Table 2b), giving a further 60 individual plates. In total, this experiment consisted of 220 individual test plates.

We transferred 2.31 \times 10^{5} cells (to reach a proportion of 350 cells \cdot \text{mm}^{-2}) to each 14 cm petri dish containing 100 mL of culture medium. The dishes were then incubated for 14 d in a thermostatic chamber (Angelantoni s.p.a., Massa Martana, Italy, model PE2611T8). For the test of sealing effect, 20 petri dishes for each species and each neon lamp were sealed by a Parafilm® foil wrapped around the dish, isolating the content and preventing any gas exchange. This treatment also prevented any accidental introduction of bacteria (increasing the axenicity) and reduced evaporation. After the 14 d period, each dish was examined under inverted microscopy (Leica Microsystems Srl, Milano, Italy, Leica DME5000) to determine the mean number of cells for each square millimeter. To obtain these data, 10 random counts were recorded for an area of 0.01 mm². The counts recorded in all dishes were averaged to calculate the final number of cells \cdot \text{mm}^{-1}, according to each treatment. After the evaluation of the numerical abundance of diatoms, the dishes were washed twice with 20 mL of distilled water, then frozen, and lyophilized. Their dried content was then scraped by means of a blade and weighed, to determine total biomass.

Weight productions. To increase the number of samples available for biomass data, 250 additional petri dishes for each treatment were inoculated and cultured in 1/2 medium to evaluate the weight production. In this case, after 14 d dishes were washed twice with 20 mL of distilled water, then emptied and frozen, to be lyophilized and weighed.

Growth dynamics. Finally, an experiment was devised to compare the growth patterns of the two species in S and NS dishes, as well as under different light spectra. For this purpose, 20 replicate dishes were inoculated for each strain (C1 and C2) and in each condition (S and NS) giving a total of 80 test plates. In addition, 20 dishes were cultured for each species (in NS conditions) and for each of two neon lamps (Aq and GL), giving a total of 80 test plates. Finally, 20 replicates were performed for each growth medium (1/2, K, and MJ) and each strain (C1 and C2), giving a total of 120 test plates. This gave a total of 280 individual test plates. Cell counts were performed as reported above (10 random counts on 0.01 mm² areas for each dish), but in this case, they were repeated at 3 d intervals between days 0 and 15, giving a total of five sets of cell counts for each dish. Growth curves were then obtained and plotted according to the relationship:

\[
y = \frac{a}{1 + e^{-\left(\frac{x-b}{c}\right)}}
\]

using GraphPad Prism® V. 3 software (GraphPad Software Inc., La Jolla, CA, USA). This allowed us to plot the growth curves for each experimental condition (Davidson 2002). Also in this case, at the end of the experiment and after the last cell count, each dish was washed twice with distilled water and dried, to record the weight production, to be compared with the other data previously described. Therefore, growth curves were obtained for each of two culture conditions (S vs. NS), each species (C1 vs. C2), each of three culture media (1/2, K, and MJ), and two neon lamps (Aq vs. GL).

Growth rates (GRs) were also calculated for each condition, at each time lag, using the following formula (Parker et al. 2007):

\[
GR\ (\%\text{d}^{-1}) = 100 \times \frac{\ln(\text{final no. cells mm}^{-2}) - \ln(\text{initial no. cells mm}^{-2})}{\text{no. days}}
\]

In addition, cell doubling time (μ) was calculated in each condition according to the following formula (Wood et al. 2005, de la Peña 2007):

\[
\mu = \frac{\ln(\text{final no. cells mm}^{-2}) / (\text{initial no. cells mm}^{-2})}{\text{no. days}}
\]

The main growth parameters (i.e., duration of the lag phase and of the exponential growth phase, maximum density reached at the stationary phase, daily GRs, and weight gains) obtained in each condition (according to culture media, light spectrum, and experimental conditions) were finally recorded in a matrix “growth parameters versus culture conditions” submitted to correspondence analysis (C.A.) using Statistica ver. 8 software (Statsoft Inc., Tulsa, OK, USA), to detect how each growth parameter was influenced by individual factors.

Correspondence analysis is an exploratory technique used to analyze multifactorial matrices. The results provide information that is similar in nature to those produced by other factor analysis techniques, and they allow one to explore the structure and the relationships of categorical variables included in the matrix (Lopes et al. 2010). The final goal of C.A. is to find theoretical interpretations for the extracted dimensions. In our case, a matrix of five growth parameters by eight culture conditions was analyzed, and the first two factors were plotted.

Confirmation of the bioactivity. Since the aim of diatom cultivation may be represented by the production of bioactive substances, we tested the diatoms produced for their content of an apoptogenic compound able to influence the sex reversal of a decapod crustacean. The results of these tests are out of the aims of the present investigation, focused on the influence of various culture factors on the growth of a benthic diatom. However, to confirm that the most productive conditions did not hamper the ability of diatoms to produce secondary metabolites, we performed specific tests whose methods and results are reported in Zupo and Messina (2007).

RESULTS

There was no significant difference in productivity between the two strains (P > 0.05). *C. scutellum scutellum* produced on average 5.77 ± 1.36 mg dwt of biomass per dish, while *C. scutellum parva* produced on average 5.72 ± 1.33 mg dwt of biomass per dish. The diatom biomass obtained was tested for apoptogenic activity, and presence of the active metabolite was confirmed (see Zupo and Messina
However, large differences were detected in terms of both number of cells produced at the end of the experiment and dwt gain in relation to variation of the selected factors (Fig. 1).

Taking into account the first part of the experiment (Table 2a), conducted using f/2 medium, the highest final density (22,820 cells \( \cdot \) mm\(^{-2} \) \cdot \) dish\(^{-1} \)) was recorded with the cultivation of *C. scutellum scutellum* in NS conditions, under AS light (Fig. 1a). Overall, NS dishes exhibited higher densities (13,730 cells \( \cdot \) mm\(^{-2} \) \cdot \) dish\(^{-1} \) ± 1,504) in comparison to sealed ones (10,438 cells \( \cdot \) mm\(^{-2} \) \cdot \) dish\(^{-1} \) ± 3,631) under all experimental conditions, unaffected by light spectrum and strain. Accordingly, NS dishes showed higher mean final dry weights (5.06 ± 3.28) in comparison to sealed dishes (4.46 ± 1.15; Fig. 1b).

In terms of light source influence, the highest average density (18,695 cells \( \cdot \) mm\(^{-2} \) \cdot \) dish\(^{-1} \)) was obtained under AS light, followed by Aq (11,770 cells \( \cdot \) mm\(^{-2} \) \cdot \) dish\(^{-1} \) ± 8,032) and GL (9,758 cells \( \cdot \) mm\(^{-2} \) \cdot \) dish\(^{-1} \) ± 3,873). However, these data were not consistent with dwt gain (Fig. 1b). Surprisingly, the highest average weight (5.2 mg \cdot \) dish\(^{-1} \) ± 0.71) was obtained under GL light, followed by AS light (5.15 mg \cdot \) dish\(^{-1} \) ± 1.34) and Aq (3.80 mg \cdot \) dish\(^{-1} \) ± 0.56). Therefore, GL promotes the lowest density and the highest weight gain. This relationship may indicate that GL promotes the production of larger cells.

Significant differences were found for each treatment condition (Table 3). Light spectrum, strain, and culture conditions (S vs. NS) all influence *C. scutellum* production (at \( P < 0.01 \)). Overall, ANOVA indicates that *C. scutellum scutellum* (C1) exhibited higher GRs in sealed dishes when lit by GL than *C. scutellum parva* (C2). In contrast, C2 performed better than C1 in NS conditions when lit by GL and Aq (Fig. 2). C1 exhibits the best performance under Aq, both in S and NS conditions. Therefore, C1 and C2 exhibit contrasting performances under S and NS conditions, when lit by GL. In fact, C2 produces much more than C1 in NS conditions, while it produces less than C1 in sealed conditions, when lit by GL, according to a multivariate growth strategy.

Significant differences were seen between culture media for final cell density and weight gain. MJ promoted the highest weight gain (10.74 mg \cdot \) dish\(^{-1} \) ± 0.9; Fig. 3b), as well as the highest GRs (Table 4). Surprisingly, f/2 (the most commonly used medium) produced the lowest final density (15,420 cells \( \cdot \) mm\(^{-2} \) \cdot \) dish\(^{-1} \) ± 3,691) and lowest weight gain (5.70 mg \cdot \) dish\(^{-1} \) ± 0.8) (Fig. 3).

Experimental factors showed a clear influence on the growth curves of the two strains (Fig. 4). In NS conditions, *C. scutellum scutellum* reaches exponential growth on the 9th day of culture (Fig. 4a), while this phase starts on the 5th day in S dishes (Fig. 4c) and the stationary phase is reached earlier, but it is characterized by higher final densities (872 cells \( \cdot \) mm\(^{-2} \)). In contrast, *C. scutellum parva* reaches the stationary phase in comparable periods in S and NS dishes (Fig. 4, b and d), but it is characterized by significantly higher densities (no. cells \( \cdot \) mm\(^{-2} \)) and higher GRs (Table 4) in NS dishes. Cell doubling (\( \mu \)) \( d^{-1} \) rates were relatively low: the maximum rate was 2.34, reached by *C. scutellum parva* in NS conditions under GL lamp in MJ medium. *C. scutel-

![Figure 1](image)

**Fig. 1.** Final production obtained in f/2 medium, recorded at the 14th day of experiment according to the variations of several factors (NS, nonsealed; S, sealed; Aq, Philips Aquarelle; AS, Sylvania Aquastar; GL, Sylvania Gro-Lux). (a) Final number of cells per surface unit; (b) dry weight (dwt) obtained per dish. *C., Cocconeis.*

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>MS</th>
<th>( F )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>1</td>
<td>1.17E+10</td>
<td>77.3</td>
<td>0.000</td>
</tr>
<tr>
<td>Light</td>
<td>2</td>
<td>1.73E+10</td>
<td>115.5</td>
<td>0.000</td>
</tr>
<tr>
<td>Condition</td>
<td>1</td>
<td>6.34E+09</td>
<td>42.2</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**Table 3.** Analysis of variance (ANOVA) table indicating the main results of the analysis according to diatom strain (C1, C2), light spectrum (Aquastar, Aquarelle, Gro-Lux), and culture conditions (sealed or nonsealed dishes), following the experimental plan of Table 2a.

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C. scutellum exhibited, on average, lower doubling rates. Highest values occurred mainly between the 6th and the 9th day of culture with few exceptions (represented by the media MJ and K, in which \( \mu \) keeps high values up to the 12th day) with mean values of 1.94 for C. scutellum scutellum and 2.02 for C. scutellum var. parva.

As for the effect of light on the growth curves, C. scutellum scutellum reached similar values in the stationary phase, when lit by Aq lamps (Fig. 4e) and GL (Fig. 4a), but the log phase (exponential growth) was reached earlier under Aq lamps. C. scutellum var. parva, in contrast, did not exhibit significant differences when lit by Aq lamps (Fig. 4f) and GL (Fig. 4b), although under the latter light the stationary phase was characterized by slightly lower numerical abundance per surface unit. This datum was consistent with GRs (Table 4), indicating that Aq lamps promote an earlier production burst but lower average productivity.

Finally, when the effect of culture media is tested (Fig. 4g) it is evident that K and MJ media produce very similar growth patterns, with a log phase that started on the 9th day of culture. In contrast, \( \frac{1}{2} \) medium produced an earlier start to the log phase (6th day) but a slightly lower cell concentration at the stationary phase. GRs (Table 4) were significantly different (\( P < 0.001 \)) between treatments over time. In all treatments, the GR was highest between days 3 and 9, although its level was kept high in MJ medium, up to the 12th day.

The C.A. (Fig. 5) performed on Table 5 yielded three clusters partially overlapped in the center of the factorial plane (defined by F1 and F2). The first one, in the second quadrant, indicates the relationship between the main factors, that is, culture media and light spectrum, and the maximum density reached in the plates. The second one, in the first quadrant, indicates that sealing of dishes influences the GR and the duration of the lag phase. The third cluster, in the center of the first two quadrants, contains the duration of the exponential growth phase along with the S and NS conditions. The final weight gain occupies a polar position in the third quadrant. The main relationships between culture conditions and growth parameters, emerging from the analysis of growth curves (Fig. 4) and C.A. (Fig. 5) are summarized in Table 6.

**DISCUSSION**

Our results indicate that C. scutellum can grow under both S and NS conditions and use a varied light spectrum, which suggests this species is well adaptable to a range of ecological conditions and it may take advantage of multiple regimes in a changing environment (Thessen et al. 2009). Since previous investigations (Raniello et al. 2007) have indicated, for congenic diatoms, the optimal irradiance levels to be between 60 and 140 \( \mu \text{M photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \), and all our tests were conducted at similar PAR, an influence of light intensity on the productivity of our cultures may be excluded. These low light intensities are in accordance with the results obtained by Parker et al. (2007) for Cocconeis sublittoralis and confirm that most Cocconeis species grow preferentially in shaded environments. In addition, this irradiance range has been shown to be suitable for most epibenthic diatoms (Lewis et al. 2002, Mouget et al. 2005), and Cocconeis spp. are
considered to be dominant in subtidal regions (Round 1971). We cannot exclude that an increased irradiance, producing metabolic stress, could induce an increase in the production of secondary metabolites (Mouget et al. 1999). However, this variable was not investigated in the present research, since we tested the effect of various spectra at a constant level of irradiance.

The two considered varieties of *C. scutellum* exhibited contrasting physiological results to the given sets of experimental parameters (Wood and Leathem 1992). In fact, we demonstrated that C1 produces less than C2 under GL light, when cultured in NS conditions, but it produces more than C2, in the same environment, when cultured in sealed conditions. Also, the cultures obtained under an Aq spectrum reach a higher density in NS conditions, while the benefit of Aq light is low (Aq and GL production became comparable) when the diatom is cultured in sealed conditions. This may be due to different cell quotas characterizing the two species (Davidson and Gurney 1999) and appears to make sense, considering that the sealing of dishes may be viewed in terms of carbon limitation (CO₂ depletion due to reduced gas exchange). Carbon limitation is known to produce stress on the metabolism of microalgae (Morel et al. 1995) and influences important features of the growth process, such as duration of the log phase, GR, and weight gain (Table 6). *C. scutellum* strains exhibited differences in these characters, producing contrasting physiological results.

Growth rates and final densities obtained, however, are not always in accordance with weight gains. The differences may indicate an effect on cell volume, which in diatoms may also be referred to as the frequency of sexual reproduction (Assmy et al. 2008). In fact, during the asexual phase the cell volume decreases at each division, and a larger size is restored after a sexual reproduction event (Jewson 1992). Therefore, some light spectra (e.g., GL) could elicit a reduction in the number of asexual divisions, or stimulate successful sexual events, which could explain the lower number of cells per surface unit, along with the higher weight reached at the end of the experiment (Montresor and Lewis 2005). This point is worth noting, since several articles evaluated the GR and the efficiency of the production of diatoms based exclusively on the rate of cell division (Davidson et al. 1999). This study demonstrates that the biomass production, in cul-

### Table 4. Growth rates (no. cells/no. days × 100) determined during the culture period, for each of the considered culture conditions. C1, C2 = diatom varieties. NS, S = nonsealed, sealed conditions; GL, Aq = neon lights used, that is, Sylvania Gro-Lux lamp; K, Keller culture medium; MJ, modified Jørgensen culture medium.

<table>
<thead>
<tr>
<th>Days</th>
<th>C1 NS GL</th>
<th>C1 S GL</th>
<th>C1 NS Aq</th>
<th>C2 NS GL</th>
<th>C2 S GL</th>
<th>C2 NS Aq</th>
<th>C2 NS GL K</th>
<th>C2 NS GL MJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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</tr>
<tr>
<td>3</td>
<td>8.75</td>
<td>8.75</td>
<td>3.98</td>
<td>1.63</td>
<td>11.69</td>
<td>17.03</td>
<td>6.08</td>
<td>3.33</td>
</tr>
<tr>
<td>6</td>
<td>19.11</td>
<td>33.83</td>
<td>27.75</td>
<td>51.95</td>
<td>49.63</td>
<td>37.58</td>
<td>66.41</td>
<td>67.27</td>
</tr>
<tr>
<td>9</td>
<td>2.55</td>
<td>16.06</td>
<td>21.21</td>
<td>26.11</td>
<td>11.26</td>
<td>24.25</td>
<td>31.70</td>
<td>19.54</td>
</tr>
<tr>
<td>12</td>
<td>1.90</td>
<td>0.06</td>
<td>4.13</td>
<td>0.00</td>
<td>1.41</td>
<td>0.36</td>
<td>1.60</td>
<td>0.00</td>
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<td>15</td>
<td>1.11</td>
<td>1.02</td>
<td>5.88</td>
<td>0.00</td>
<td>1.41</td>
<td>0.36</td>
<td>1.60</td>
<td>0.00</td>
</tr>
</tbody>
</table>

C1, *Cocconeis scutellum* var. *scutellum*; C2, *Cocconeis scutellum* var. *parva*; f/2, Guillard’s f/2 culture medium; K, Keller culture medium; MJ, modified Jørgensen culture medium.
Fig. 4. Growth curves obtained over 15 d of culture according to diatom variety and factors considered (f/2, K, and MJ are culture media; Aq, GL, and AS are lights; NS and S are sealed or nonsealed). Error bars refer to differences among cell counts obtained in the same day. Aq, Philips Aquarelle lamp; AS, Sylvania Aquastar lamp; f/2, Guillard’s f/2 culture medium; GL, Sylvania Gro-Lux lamp; K, Keller culture medium;MJ, modified Jørgensen culture medium.
Fig. 5. Results of the correspondence analysis (C.A.) performed on the matrix “growth parameters vs. culture conditions.” The first two factors are plotted. Aq, AS, and GL are neon types (as in Fig. 1). MJ, K, and $f^2$ are culture media (according to Fig. 3). S and NS are culture conditions (sealed or nonsealed). Spd = lag phase duration; EPD = exponential growth phase duration; Max = maximum density reached (no. cells $\cdot$ mm$^{-2}$) at the stationary phase; GR = growth rate; W = weight gain at the end of the test. Growth parameters are printed in gray color, and they are indicated by large circles. Culture conditions are printed in black, and they are indicated by small circles. Ellipses containing the three main clusters are discussed in the text. Aq, Philips Aquarelle lamp; AS, Sylvania Aquastar lamp; $f^2$, Guillard’s $f^2$ culture medium; GL, Sylvania Gro-Lux lamp; K, Keller culture medium; MJ, modified Jörgensen culture medium.

Table 5. Results obtained in each treatment, averaged among replicates for each experimental condition and used for the correspondence analysis. $f^2$, K, and MJ are culture media. GL, Aq, and AS are neon lamps. NS and S are culture conditions (nonsealed and sealed, respectively). GR indicates growth rate.

<table>
<thead>
<tr>
<th>Parameter treatment</th>
<th>Medium</th>
<th>Light</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>f$^2$ K MJ GL Aq AS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stationary phase duration (d)</td>
<td>5.33 7.00 8.00</td>
<td>5.50 4.50 4.00</td>
<td>5.75 5.00</td>
</tr>
<tr>
<td>Exponential phase duration (d)</td>
<td>2.00 3.00 3.00</td>
<td>2.00 2.50 2.50</td>
<td>2.75 1.50</td>
</tr>
<tr>
<td>Max density reached (cells $\cdot$ mm$^{-2}$)</td>
<td>1,520.00 1,800.00 1,800.00 947.50 1,130.00 1,300.00</td>
<td>947.50 1,130.00 1,300.00 1,034.00 940.00</td>
<td></td>
</tr>
<tr>
<td>Maximum GR (% d$^{-1}$)</td>
<td>51.95 66.41 67.27</td>
<td>51.95 37.58 44.77</td>
<td>67.27 49.63</td>
</tr>
<tr>
<td>Weight gain (mg dwt $\cdot$ dish$^{-1}$)</td>
<td>5.70 8.90 10.74</td>
<td>5.20 3.80 5.15</td>
<td>5.06 4.46</td>
</tr>
</tbody>
</table>

Aq, Philips Aquarelle lamp; AS, Sylvania Aquastar lamp; dwt, dry weight; $f^2$, Guillard’s $f^2$ culture medium; GL, Sylvania Gro-Lux lamp; K, Keller culture medium; MJ, modified Jörgensen culture medium.

Table 6. Effect of the factors considered (in rows) on the growth descriptors of diatoms (in columns). The main relationships found, synthesizing the results of the study of the growth curves, the factorial tests and the analysis of correspondences are checked.

<table>
<thead>
<tr>
<th>Lag phase duration</th>
<th>Exponential growth (log phase) duration</th>
<th>Max cell density at stationary phase</th>
<th>Metabolite production</th>
<th>GR</th>
<th>Weight gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture media</td>
<td>$\checkmark$</td>
<td>$\checkmark$</td>
<td>$\checkmark$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scaling of dishes</td>
<td>$\checkmark$</td>
<td>$\checkmark$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diatom variety</td>
<td>$\checkmark$</td>
<td>$\checkmark$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light spectrum</td>
<td>$\checkmark$</td>
<td>$\checkmark$</td>
<td>$\checkmark$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GR, growth rate.
tarture, may be relatively distinct from the GR and the final density. Therefore, caution must be exercised when applying even simple models to conditions other than those in which they were derived (Davidson and Gurney 1999). We should also consider that a relationship exists between the ratio of secondary metabolite production and cell size, peaking in Haslea ostrearia in the range of 55–70 μm (Mouget et al. 2005). Therefore, this is an additional factor potentially able to influence the production of bioactive compounds, since evidence for selection against sexual reproduction has been seen in diatoms in culture, which often display reduced sexuality with time (Chepurnov et al. 2004).

The GRs obtained in NS dishes are higher under Aquastar spectrum (AS) than those obtained under GL and Aq. In fact, AS lighting of NS dishes produced 22,820 cells · mm⁻² of C. scutellum scutellum per dish, as compared to 6,870 (GL) and 6,090 (Aq). In this case, the GRs are in agreement with the weight gains because AS used for NS dishes yielded 6.1 mg dwt per dish, compared to 5.9 mg (GL) and 3.4 mg (Aq). Therefore, significantly higher GRs were obtained using an AS spectrum, in NS conditions, when l/2 medium was employed.

The effect of sealing is more complex and is dependent on the strain and the spectrum applied, indicating that each variety needs a given set of physical and chemical variables to maximize production (Thessen et al. 2009). However, the two considered varieties produced, in the same experimental conditions and over the same time period, similar biomasses (4.6 mg of C1 vs. 4.9 mg of C2 per dish), although the GR of C2 is approximately double (final mean density: 13,540 cells · mm⁻²) the GR of C1 (final mean density: 7,318 cells · mm⁻²). This finding is due to the lower cell size characterizing the strain of C. scutellum parva in comparison to C. scutellum scutellum (De Stefano et al. 2008).

The effect of the culture media, in contrast, is negligible when considering the GR, but an important factor in the outcome of dwt production. The three culture media gave similar final cell densities (17,231 cells · mm⁻² ± 1,594), but dwt production varied significantly between media (10.74 mg · dish⁻¹ for MJ, 8.90 for the K, and 5.70 mg for the l/2 medium). Therefore, the l/2 medium, commonly used to cultivate these diatoms, was demonstrated to be the least efficient, while the MJ medium was confirmed to be an ideal substrate for the production of diatoms (Barsanti and Gualtieri 2006). In addition, benthic diatoms appear to possess, in comparison to planktonic ones, a certain degree of physiological inertia when ecological conditions change (Defew et al. 2002). Therefore, the effect of culture media could be more pronounced when strains are cultivated for longer periods in K or MJ media.

The examination of the growth curves along with the results of the C.A. helps understanding these relationships and shows that culture media are primarily responsible for the determination of the duration of the lag phase and the maximum cell density reached at the stationary phase (Table 6). In fact, comparisons of the three media for the culture of C. scutellum parva indicated a lag of 6 d to reach the exponential growth phase, and this time lag was common to most experiments in various conditions of light and gas exchange, with the exception of C. scutellum scutellum in NS conditions lit by GL (Fig. 4a), in which the attainment of the exponential growth phase was delayed until the 9th day. The sealing of dishes had an effect on the duration of the exponential growth phase, which was reduced in both species when gas exchange was limited. In these conditions, diatoms have lower reserves of CO₂ available, and the exponential growth phase lasts only 1–2 d (according to the species), in comparison with a 3 d exponential growth phase in NS dishes. Finally, the maximum cell density reached in the stationary phase is primarily dependent not only on the species and variety considered (in accordance with Passy 2008), but it is also influenced by the culture medium, with MJ and K promoting the highest densities. This finding may have important implications for future research, as several authors (e.g., Parker et al. 2007) have used l/2 as a standard culture medium for most species of diatoms.

The light spectrum has a variable effect, according to the strain and other culture conditions, but could be a key factor in triggering the production of secondary metabolites (Wiegman et al. 2002). For example, it was demonstrated that the pennate diatom Haslea ostrearia, acclimated to various light spectra, exhibited almost identical growth, photosynthesis, and chl a content, but the production of an important secondary compound (the pigment marennine) was enhanced under blue light (Mouget et al. 2005). This effect has been confirmed by our observations, since GL light maximized the production of the proapoptotic compound, as demonstrated by activity tests on Hippolyte inermis (Zupo et al. 2007). In addition, the production of selected metabolites may be limited to the lag phase of growth or to the exponential phase (Brown and Miller 1992, Brown et al. 1999). Therefore, factors influencing the duration of each phase and its intensity may dramatically affect the production of biologically active compounds (Lebeau and Robert 2003b).

It is also known that stress increases the production of secondary metabolites while negatively influencing biomass production of cultures (Lebeau and Robert 2003a). In fact, GL light could represent a stress factor for C. scutellum (its spectrum is quite different from sunlight), and, as observed, it does not always promote the highest GRs but triggers an overproduction of the biologically active compounds (Zupo et al. 2007). Other stress factors, such as an increase in day length could be tested in the future. Couteau (1996) suggested, for example, that the
duration of artificial illumination should be a minimum of 18 h light · d⁻¹, and this modification could lead to both an increase in biomass production (enhanced photosynthesis) and secondary metabolites (metabolic stress). However, this extreme condition was not tested in the present investigation and is an area for consideration of future research efforts.

It was demonstrated (Table 4) that the highest GRs were consistently recorded on the 6th day of culture, unaffected by light spectrum, culture media, or other culture conditions. Therefore, this metabolic feature appears independent from the considered ecological constraints. The shape of the growth curve, therefore, is primarily due to the length and intensity of the exponential growth phase. GRs (μ) were relatively low in comparison to other diatom species (Haney and Jackson 1996, de la Peña 2007), although slow GRs have been shown to be characteristic of Cocconeis spp. (Graham et al. 2005).

In conclusion, our results indicate that C. scutellum parva, lit by GL lamps and cultured in MJ medium in NS conditions, represents the best compromise to maximize the production of the proapoptotic secondary metabolite (Zupo 2000). The production of secondary metabolites is dependent on the diatom variety and even the clone considered (Marchetti et al. 2004, Thessen et al. 2009). The effects of light spectrum, gas exchange limitation, and culture media on given features of the diatom metabolism and growth patterns represent a promising starting point for devising ideal culture conditions, with the aim of obtaining homogeneous production, in terms of quality and quantity, to be confirmed in other species of microalgae.

The experiments described herein have been conducted by Carmela Patalano in the frame of her degree thesis work. This research was funded by the European Community (004800 Pharmapox, coordinated by Dr. V. Zupo). The activities of P. Messina for this research were funded by an E.U. grant. Thanks to Dr. M. De Stefano for the taxonomic confirmation of our diatom strains. The English text was kindly revised by Rosanna Messina and Samantha Garrard. Two anonymous reviewers offered valuable suggestions to improve the quality of our manuscript.


Feeding of *Penaeus japonicus* Bate (Decapoda: Penaeidae) in pond cultures: size descriptors and food selection

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The feeding pattern of *Penaeus japonicus* Bate, cultured at different densities, with and without the addition of artificial diets, is investigated in the present paper, to identify any character of prey influencing the feeding preferences of shrimps. Biometric, calorific and gut contents data of shrimps at different developmental stages were recorded, as well as the abundance of benthic prey. A larger size is correlated with an increase in energetic value in the shrimp tissues, as confirmed by the logarithmic relationship between length and weight. Results indicated that artificial diets are chosen at the same rate in each size class and are a good source of energy for shrimps. *P. japonicus* is able to use different food sources, and its feeding preferences varied according to its size. The preferred prey were polychaetes, *Chironomus* larvae and bivalve molluscs. Young specimens, however, preferred certain items, such as copepods and *Abra ovata*, while adults also used amphipods and other mollusc species. Results may be related to the different size and shape of prey.

**KEYWORDS:** *Penaeus japonicus*, culture, food selection, calorimetry, growth.

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**Introduction**

*Penaeus japonicus* Bate is a penaeid shrimp (Decapoda: Natantia), which was introduced into Europe for aquaculture purposes because of its assumed adaptability to the environmental conditions of the Mediterranean area (Lumare and Palmegiano, 1980; Lumare, 1986). It is semi-extensively cultured in Italy and in other Mediterranean countries (e.g. France, Greece). Artificial diets appear ineffective in promoting sufficient growth and survival rate (Lumare et al., 1986); however, our information about prey-preferences and feeding activity is incomplete. In particular, the prey-choice mechanism and its relationship to the environment and the density of culture is not known, neither are tactile and taste cues, that may influence the attractiveness of artificial diets. The present investigation was aimed at the identification of any character of prey (e.g. shape and size) influencing the feeding preferences of shrimps and to the assessment of biometric and calorimetric changes in the shrimps, during the grow-out period, triggering changes in their feeding choices.
The feeding activity of shrimps, cultured at different densities, was investigated for 130 days by means of gut content analyses.

Materials and methods

Five ponds were arranged in an experimental plant close to the Lesina Lagoon, Italy (figure 1), with a sandy bottom about 25 cm thick. The average water depth in the ponds was 90 cm and an amount of water ranging from 10 to 30% of the total pond volume was changed daily, according to the pond ecological conditions, the temperature and the rate of evaporation. The ponds were fertilized by chicken manure (200 kg/ha), ammonium nitrate (20 kg/ha) and calcium phosphate (10 kg/ha), at the start of the experiment. *P. japonicus* post-larvae (27 days post metamorphosis; average total length = 16.2 mm; average fresh weight = 0.042 g) were stocked at different densities after seven days from the experiment start. The largest pond (surface area 200 m²), was stocked at a density of one specimen per square meter, without the addition of artificial diets. The other four ponds (surface area 100 m²) were managed with the addition of artificial diets in pellets (Kanazawa *et al.*, 1977; Deshimaru and Yone, 1978) according to a dietary schedule described by Lumare (1988), i.e. supplying daily amounts of food ranging from 200% (initial grow-out period) to 2% (final grow-out period) of the shrimp body weight. Two ponds were managed at a density of 2.5 specimens per square meter, and the other two at a density of 1.5 specimens per square meter. The experiment lasted 130 days.

The main physical and chemical water quality parameters (temperature, salinity, pH, oxygen, total ammonia, nitrite and nitrate content, organic carbon and chlorophyll content) were monitored to verify the correspondence of environmental conditions to the physiological requirements of the shrimps. Three replicate samples of zoobenthos were collected from each pond on day 42, day 84 and day 128 of the

![Fig. 1. The experimental plant close to the Lesina Lagoon (Foggia, Italy).](image-url)
experiment by an Ekman-Birge bucket, and fixed in buffered formol. The abundance
of each species in the samples was recorded, to be compared with the prey detected
in the gut contents.

Three sample batches (five shrimps per pond) were collected from each pond on
day 42, day 84 and day 128 of the experiment. Samples were deep frozen (−40°C)
to prevent digestion of prey. Each specimen was then defrosted, measured for the
main biometric parameters (total length, from the tip of rostrum to the end of
telson; carapace length, from the tip of the rostrum to the posterior medial notch;
rostrum length, from the tip of the rostrum to the first rostral spine; first cheliped
length; fresh weight) and dissected. A logarithmic function was used to test the
correlation between shrimp weight and total length.

Guts were weighed and fixed in 70% alcohol, after dissection. Gut contents were
examined to identify prey to the lowest possible taxonomic level. The abundance of
each item was coded by a 0 to 5 index, to obtain a matrix ‘shrimps vs. food items’.
Each item was then classified into four subjective categories: globose and large (GL);
globose and small (GS); elongated and large (EL); elongated and small (ES). The
terms ‘small’ and ‘large’ are prey size descriptors (table 1). Therefore, a matrix
‘shrimp vs. prey category’ was obtained.

The body of each specimen was homogenized, freeze dried and weighed; a small
sample was dried at 105°C until a constant weight was reached, and then newly
weighed to record the residual water content. Two calorimetric tests were performed
on each specimen by a ‘JKA-C400’ type micro-bomb calorimeter, to determine the

Table 1. Prey items found in the gut contents, classified according to their shape and size.
Prey having, in the gut contents, the largest dimension higher than 3 mm were consid-
ered ‘large’; prey having, in the gut contents, the largest dimension lower than 3 mm
were considered ‘small’. Prey whose largest dimension was more than 3 times its
smallest dimension was considered ‘elongated’; prey whose largest dimension was less
than 3 times its smallest dimension was considered ‘globose’.

<table>
<thead>
<tr>
<th>Globose-Small</th>
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<tbody>
<tr>
<td>Foraminiferids</td>
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<tr>
<td>Eggs of insects</td>
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<tr>
<td>Copepods</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Globose-Large</td>
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<tr>
<td>Gastropods</td>
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<tr>
<td>Abra ovata</td>
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<tr>
<td>Cerastoderma glaucum</td>
</tr>
<tr>
<td>Gammarid amphipodes</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Elongated-Small</td>
</tr>
<tr>
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<tr>
<td>Cladophora sp.</td>
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<tr>
<td>Chaetomorpha gracilis</td>
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<tr>
<td>Chaetomorpha capillaris</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Elongated-Large</td>
</tr>
<tr>
<td>Polydora ciliata</td>
</tr>
<tr>
<td>Other polychaetes</td>
</tr>
<tr>
<td>Chironomus larvae</td>
</tr>
<tr>
<td>Other insect larvae</td>
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<tr>
<td>Isopods</td>
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</tbody>
</table>
calorific value of shrimp tissues. The instrument was calibrated by benzoic acid. Results were recorded in a matrix 'Specimen-calorific data'.

Differences among data obtained in the five ponds, cultured at different densities, were tested by ANOVA. Data matrices obtained in each pond, concerning biometric, calorimetric and gut content data on shrimps were analysed by correspondence analysis (Benecki, 1980) and the significance of axes was tested by the method proposed by Frontier (1974). This technique of multivariate analysis is based on the assumption that correlations among variables under investigation may be due to the presence of underlying (or latent) factors. Variables are then 'ordered', projecting the N 'observation' points in the space defined by an S 'variable' coordinate frame, onto a space of fewer dimensions, in such a way that the arrangement of the points undergoes the least possible distortion, thus preserving the most important features of the original S-space patterns. All axes are orthogonal as we wish the new variables (factorial variables) to be uncorrelated. The resulting ordination model allows an easy visualization of these patterns in two or more dimensions (factors) that are explanatory of a given portion of the system total variance. Factors do not correspond to the classical notion of ecological factors, but rather to a combination of highly correlated characteristics of the system under investigation (McKeon, 1966; Masson, 1974).

Results

During the experiment the main parameters of water quality were in the range considered suitable for shrimp development, according to Lumare et al. (1986). The taxonomic investigations of macrozoobenthos indicated no significant difference (ANOVA) between the replicates obtained in each pond. The total number of individuals detected in benthos samples was significantly and inversely correlated (r = 0.8) to the density of shrimps at the end of the experiment. Slight differences in benthic associations were detected in each pond. However, the differences were not significant (ANOVA) in the first four ponds, cultured with addition of artificial diets. The dominant groups, during the grow out period, were polychaetes (represented mainly by Polydora ciliata (Johnston)), molluscs (represented mainly by Abra ovata (Philippi)) and Cerastoderma glaucum (Poiret), harpacticoid copepods and insect larvae (figure 2).

No significant differences were found in the biometric characters (fresh and dry weight of shrimps, fresh weight of guts, carapace, first cheliped, rostrum and total length) of shrimps cultured in the five ponds. Therefore, the average biometric measures obtained in the five ponds will be reported. Total length of shrimps cultured in the five ponds, in the three samples, ranged from 8.3 to 16.8 cm; their weight ranged from 3.7 to 37.2 g; carapace length from 3.2 to 6.5 cm; rostrum length from 1.9 to 3.8 cm; first cheliped length from 1.1 to 2.6 cm; stomach weight from 0.05 to 0.33 g. Shrimp weight was significantly correlated to their length (R² = 0.98; figure 3A) by a logarithmic relationship. Dry and fresh weight of shrimps were linearly and significantly correlated (R² = 0.98; figure 3B). The correlation between shrimp size (both in terms of weight and length) and gut weight was not significant, although dry weight of shrimps was significantly related to all the other biometric characteristics. The highest correlation values were exhibited by carapace length and dry weight of shrimps (r = 0.92), total length and carapace length (r = 0.92), rostrum length and carapace length (r = 0.92), carapace length and first cheliped length (r = 0.89), first cheliped length and stomach weight (r = 0.80). The mean length of
shrimps from all the ponds, in the first sample, was 9.4 ± 0.6 cm; in the second 13.4 ± 0.63 cm; in the third 15.6 ± 0.7 cm.

The main food items in the stomachs of shrimps from all the ponds were polychaetes (found in 61.1% of the specimens, mainly represented by *Polydora ciliata*), molluscs (55.5%, mainly represented by *Cerastoderma glaucum* and *Abra ovata*), *Chironomus* larvae (88.8%), amphipods (48.1%, mainly represented by *Gammarus aequicauda* (Martinov)). In shrimps sampled in ponds managed with
addition of artificial diets, dry food (74.2%) was also detected. Correlations between abundance of molluscs and algae in the gut content vs. shrimp size were not significant. The other items were significantly correlated to shrimp size. In particular polychaetes, *Chironomus* larvae and amphipods were directly correlated, while copepods were inversely correlated to shrimp size.

During the whole grow-out period (average data of the three sample batches), insect larvae were the most consumed prey both in ponds managed without addition of artificial diets (31%; figure 4A), and in ponds managed with addition of artificial diets, at 1.5 ind./m² density (29%; figure 4B) and 2.5 ind./m² density (33%; figure 4C). Also bivalve molluscs were largely consumed (26%) in ponds managed without addition of artificial diets (figure 4A), followed by amphipods (16%), copepods (especially in the first period; 12%), polychaetes (10%), and algae (5%). In ponds managed with the addition of artificial diets, shrimps cultured at density of 1.5 ind./m² (figure 4B) preferred, after insect larvae, algae (21%), polychaetes (18%), amphipods (16%) and bivalve molluscs (10%). A similar trend was exhibited by the gut contents of shrimps cultured at 2.5 ind./m², with insect larvae followed by
Fig. 4. Percentage of different prey in the gut content of shrimps cultured in ponds managed without (A), and with addition of artificial diets at density of 1.5 (B) and 2.5 (C) individuals per square meter. Average data for the three samples are given.

amphipods (24%), bivalves (20%), polychaetes (11%) and algae (9%). The differences between percentages of different prey consumed in ponds managed with and without addition of artificial diets were not significant at $P<0.01$. Copepods, amphipods and the bivalve molluse *Abra ovata* exhibited a selective presence pattern, respectively, in the young specimens and in adults; polychaetes and *Chironomus* larvae were present in the diet of both young and adult specimens.
The correspondence analysis, performed on the vertical linkage of the two data matrices, yielded only one significant axis accounting for 65.4% of the system variance. The first axis had mainly the contribution of the variable ‘fresh weight’; the second that of ‘dry weight’; therefore, it may be assumed that the ordination is given in a size range. The two parameters are significantly correlated ($R^2 = 0.98$). Therefore, the ordination model can be resumed by projecting the observation points on a single axis, as shown in figure 5. The ordination model given by this analysis showed an unimodal trend, in which food items were aligned from the item ‘Copepods’, linked to *Abra ovata* and insects, to amphipods. *Cerastoderma glaucum* and all the other observation points were collapsed in a central position. The preference pattern of shrimps of different size was revealed. Copepods, the bivalve *Abra ovata* and insect larvae were the most consumed prey in the first period of culture. *Chironomus* larvae, the algae *Chaetomorpha* sp. and *Cladophora* sp. and the polychaete *Polydora ciliata*, respectively, were the most consumed prey in the second sample. Amphipod crustaceans and the bivalve *Cerastoderma edulis* were mainly consumed in the last grow-out period. Most of the specimens were centrally ordered in the ordination model, while food items were positioned according to the size classes. Most items were used by all age classes, although young specimens preferred some items such as copepods and *Abra ovata*, and adults used prey such as amphipods and some molluscs. Artificial diets, ordered in a central position, were used at the same rate by all size classes.

The mean calorific value of shrimps was 4784 cal/g ($s = \pm 170; e = \pm 21; n = 75$), ranging from 4647 cal/g in the first sample, to 4724 cal/g in the second sample, to 4911 cal/g in the third sample (figure 5). The instrumental mean error was less than 68 cal/g. Calorific value and total length of shrimps were correlated to some preferred prey (figure 5).

![Diagram](image)

**Fig. 5.** Main biometric results. The gut content points obtained by the ordination model are projected on the first axis (F1). Bars in the positive field represent the energetic content of shrimps in each sample; bars in the negative field represent their average length in each sample.
The study of the matrix 'shrimps vs. food item categories' indicated that mainly small prey were consumed in the first period of the grow-out, and the prey size increased during grow-out. Prey size represented the main factor of choice. Elongated prey were generally preferred to globose prey (figure 6). The correspondence analysis performed on this matrix (figure 7) revealed two significant axes. 'EL' occupied a

![Graph showing abundance of different prey categories](image)

**Fig. 6.** Percent abundance of the different prey categories in the gut content of shrimps of the three samples: ES = elongated and small; EL = elongated and large; GS = globose and small; GL = globose and large.

![Correspondence analysis](image)

**Fig. 7.** Correspondence analysis of the matrix 'prey categories vs. shrimps'. Both the clustering of shrimp samples and the position of prey categories are indicated in the factorial space defined by the first two axes (both significant). Axes are not dimensioned, being factorial. ES = elongated and small; EL = elongated and large; GS = globose and small; GL = globose and large.
central position, thus confirming the importance of ‘elongated’ prey in the diet of shrimps. Most observation points were in the upper quadrants, linked to ‘ES’ and ‘EL’ prey. ‘GS’ and ‘GL’ prey were confined in the negative portion of F2, linked to juvenile shrimp samples. The differences between samples in ponds managed with and without addition of artificial diets were not significant but ‘small’ prey were constantly linked to the first period of grow-out.

Discussion

The significant relationships found among the main biometric parameters recorded, demonstrated that each one can be a useful size descriptor. Total body length or fresh weight may be easier to measure; however, calorimetric value, dry weight and carapace length may be taken into account and allow calculation of the other biometric measures by the equations previously reported. The previous equations demonstrated that the intermoult growth is a ‘homogeneous’ process during the life cycle, as calorimetric value and other biometric parameters were linearly correlated and not influenced by shrimp density, abundance of food or life stage. Interestingly, total length was related to the body weight by a logarithmic function, as weight increases were greater than length increases, in the last grow-out period; an inverse relationship was demonstrated in young specimens. Our results, indicating that calorific data are correlated to size increases, are in accordance with the results of Machado and Galhano (1980), on *Athyaeephyra desmaresti* Millet (Decapoda: Natantia). The calorific value may be considered as a good descriptor of growth, accounting for the whole ‘stocked energy’ and being independent from the moult cycle, which produces a discontinuous growth (Teshima and Kanazawa, 1980).

The significant correlation between shrimp density in the ponds at the end of the experiment and the abundance of prey species in the benthic communities demonstrated the influence of shrimps on benthic associations. The species of polychaetes, molluscs and insect larvae found in the gut contents are typical of brackish water systems and characterized by ‘r’ life strategy. Considering that *Penaeus japonicus* is generally cultured in brackish water systems, which are characterized by a similar assemblage of species, and that the main water parameters during the experiment were maintained in the optimal range for the shrimp culture, most results of the present paper, related to a brackish environment close to the Lesina Lagoon, can be extended to other experimental plants. The three samples are related to the main phases of the shrimp life cycle: juvenile, adult and mature for reproduction.

The shrimps demonstrated an ‘opportunistic’ feeding choice. In fact largest amounts of algae were consumed in ponds managed with addition of artificial diets, characterized by highest density and highest predation pressure, probably because of a lack of animal prey. Shrimps, however, chose their prey mainly by size: young individuals preferred small prey and adults preferred large prey. They also preferred, when available, prey characterized by elongated shape, probably because they are easier to capture and to handle by their chelipeds. Therefore, the presence of elongated prey in the pond bottoms may have a role in shrimp culture. However, when elongated prey are lacking, shrimps can use any other source of food, both living organisms and artificial diets. Insect larvae and polychaetes were the most consumed taxa, as they were abundant in the benthos throughout the grow-out period and they have the ‘preferred’ elongated shape. Also molluscs, probably due to their slow movements, were frequently captured. However, they do not represent a ‘preferred’ prey (New, 1976). Amphipods may have a role in the diet of
P. japonicus mainly in the last grow-out period and algae may be used as a diet integration, when other prey are lacking. Artificial foods in pellet may represent an integration of diet, when natural prey are lacking, as shrimps can adapt their diet to prey availability (Forster, 1976). However, shrimps prefer small and globose prey such as copepods and Abra ovata, in the first part of grow-out, elongated and large prey, such as polychaetes and insect larvae, in the adult phase, when available. The present investigation also indicates the importance of trophic chains for the shrimp culture, although the contribution of artificial diets, as both primer for the food web and energy source for shrimps, is demonstrated (Sedgwick, 1979).

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Chapter 3.5. Food as a physiology regulator in marine invertebrates

Summary

3.5.1. Strategies of sexual inversion in Hippolyte inermis Leach (Crustacea, decapoda) from a Mediterranean seagrass meadow. (Cited by 48. I.F. = 1.79)
The shrimp H. inermis exhibits an unusual strategy of sexual inversion as an adaptation to overcome problems related to predation pressure and seasonal food availability in P. oceanica. This strategy is based on two reproductive periods showing different types of females. In this study the effect of food on sexual development was not yet hypothesized.

3.5.2. Effect of microalgal food on the sex reversal of Hippolyte inermis (Crustacea, Decapoda). (Cited by 52. I.F. = 2.62)
The effect of diatoms of the genus Cocconeis on the sex reversal of the shrimp Hippolyte inermis Leach was examined in the laboratory. The results provide an explanation of the patterns observed in the field, demonstrate that microalgal food is involved into the sex change of the shrimp and are in accordance with the seasonal abundances of diatoms in the leaf stratum of Posidonia oceanica.

3.5.3. Influence of diet on sex differentiation of Hippolyte inermis Leach (Decapoda: Natantia) in the field. (Cited by 40. I.F. = 2.05)
Here for the first time an indication appears of a direct effect of a food in the sex reversal of an invertebrate, in the field. The diets of immature and adult individuals were compared to detect any influence of food on sex differentiation, since previous investigation indicated a correlation of the life cycle of this protandric species with the abundance of algal food in the environment. The influence of microalgal food on the sex reversal mechanism of this species, previously detected through laboratory experiments, was demonstrated to
control the life cycle of *H. inermis* in the field.

### 3.5.4. Do benthic and planktonic diatoms produce equivalent effects in crustaceans? (Cited by 18. I.F.= 0.91)

Various diatoms produce apoptosis both in benthic and planktonic crustaceans and their influence on the reproductive ecology and life cycles of decapods and copepods has been demonstrated. However, the effects appear deleterious for copepods and regulative for shrimp populations. This study indicates that diatom food does produce an apoptogenic effect on the two taxa and the two environments, but these effects are due to different classes of compounds and, probably, to different evolutionary patterns and relationships.

### 3.5.5. How do dietary diatoms cause the sex reversal of the shrimp *Hippolyte inermis* Leach (Crustacea, Decapoda). (Cited by 40. I.F.= 2.39)

Several congeneric diatoms influence the sex reversal of a shrimp and their activity is very specific. The ingestion of diatoms and the effect of their infochemicals are limited to a time window between the 5th and the 12th day of post-larval development.

### 3.5.6. Experimental evidence of a sex reversal process in the shrimp *Hippolyte inermis*. (Cited by 15. I.F.= 1.05)

*Hippolyte inermis* has been object of literature debates: some authors considered it as a proterandric species, other as a gonochoristic. Since this point is essential to the issue of the influence of food on its sexual maturation, this research provides a conclusive experimental evidence of the sex reversal process in this species.

### 3.5.7. Apoptogenic metabolites in fractions of the benthic diatom *Cocconeis scutellum parva*. (Cited by 15. I.F.= 4.38)

Various bioassay-directed fractionations have been performed in order to determine the apoptogenic compounds influencing the sex reversal of *Hippolyte*
inermis. The lipophilic fraction was purified, which led to the characterization of an active sub-fraction containing a highly lipophilic compound, whose molecular structure has not yet been identified. The results also point to the possible medical uses of the active compound. Once the molecular structure will be identified, the study and modulation of apoptotic processes in various types of cells will be possible.
Strategies of sexual inversion in Hippolyte inermis Leach (Crustacea, Decapoda) from a Mediterranean seagrass...

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Strategies of sexual inversion in *Hippolyte inermis* Leach (Crustacea, Decapoda) from a Mediterranean seagrass meadow

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(Received 14 May 1993; revision received 1 December 1993; accepted 11 January 1994)

Abstract

The population of the shrimp *Hippolyte inermis* Leach was investigated for one year along a transect through a bed of *Posidonia oceanica* (L.) Delile. Two reproductive periods per year were observed and two types of females were identified: one type is small, does not pass through a male stage and spawns in September to produce the next year's male generation. The other is large, passes through a male stage, and spawns in April to produce sufficient males and females for the reproductive period in September. This unusual strategy of sexual inversion could be an adaptation to overcome problems related to predation pressure and seasonal availability of food in *P. oceanica* seagrass meadows.

Keywords: Food; *Hippolyte inermis*; life cycle; *Posidonia oceanica*; Sex reversal

1. Introduction

The shrimp *Hippolyte inermis* lives in the meadows of *Posidonia oceanica* and other seagrasses (Zurique-Vázquez, 1985). It shows diel depth migrations (Cazzolino et al., 1992) and it is an important member of the food web of the *P. oceanica* ecosystem as a link between grazers and large carnivores such as decapods and fishes (Bell & Harmelin-Vivien, 1983; Zupo & Freni, 1985). Sex reversal in this decapod has been demonstrated from histological data (Reverberi, 1956) and by studies of carcinogenesis by parastichospoic (Veillet et al., 1963). From the absence of contemporaneous male and

*Corresponding author.
female gonadal buds, Reverberi (1950) concluded that sexual change in this species does not correspond to protandrous hermaphroditism (Yaldwyn, 1966). He proposed the concept of "sexual inversion due to genetic components expressed at different levels of development energy": the male gonad is expressed at low and the female gonad at high levels. However, sexual inversion, with a first phase as male and a second phase as female, is well demonstrated in this species. The objective of the present study was to clarify the influence of sex reversal on the population structure and to investigate the adaptations of the species to its environment.

In fact, the life cycle of _H. inermis_ seems to be more complex than the model proposed by Vollet et al. (1963). They reported a strategy of sexual inversion similar to that described by Noel (1973) for the shrimp _Processa edula_ (Risso), and defined a 2-yr cycle for _H. inermis_, with sexual inversion from male to female occurring during the first winter, at a size of about 12 mm. Given that sex reversal in various species of decapods has been linked to both the influence of environmental variables (e.g. temperature; Allen, 1959) and the attainment of a certain size (Noel, 1973), frequent observations of sex, size and environmental parameters are needed to accurately define the causal factor in _H. inermis_. The life history of _H. inermis_ was investigated in an attempt to clarify the strategies of sexual inversion in this species. In addition, the correspondence of sexual inversion with the annual cycle of epiphytes of the seagrass _P. oceanica_ is described.

2. Materials and methods

Samples were collected along a transect, at 1, 3, 10, 15 and 25 m, in the meadow of _P. oceanica_ off Lacco Annsa d'Iscia (Gulf of Naples, Italy), that extends from 1 to 33 m depth (Mazzzelli & Buia, 1993). At each station, two replicate samples of the vagile fauna were collected using a 0.4-mm mesh net hand-towed by a diver (Ledoyer, 1962), giving a total of 10 samples a month, from August 1981 to July 1982. This method, modified and standardized by Russo et al. (1985) and Russo & Vinci (1991), is considered to be best suited to collect vagile organisms associated with the leaf stratum (Ledoyer, 1962; Gambi et al., 1992); it is semi-quantitative and samples can therefore be compared statistically. The similarity of the assemblages of species in the two replicates at each station was evaluated by the Wilcoxon test (Sokal & Rohlf, 1973). In order to confirm the results of Wilcoxon tests, intra- and intersample similarity was evaluated by performing a mean linkage cluster analysis on the centred scalar product matrix obtained from the “size vs. stations” matrix (Oloot, 1978).

All individuals of _H. inermis_ were used, except damaged specimens where total length or sex could not be determined. The total length was measured from the tip of the rostrum to the posterior medial notch of the telson. Under a dissecting microscope (40×), each specimen was pressed against a metal ruler and the maximum extension was measured. A precision of 1 mm was considered sufficient for the purpose of the present study. Specimens were divided into 1 mm size classes.

After dissecting pleopod II, further observations were made using a compound microscope (250×). Sex was determined according to the sexual dimorphism observed.
(Fernandez-Munoz & Garcia-Rizo, 1987): the form and presence/absence of a male appendix on pleopod II. Young specimens were identified from the endopodite of pleopod II. Endopodite of pleopod II is small in immature specimens, and its shape is similar to that of pleopod I. The results, expressed for each of the five depths, were used to investigate the depth and seasonal distribution of the species in relationship to environmental changes (food and temperature). Additionally, due to the evidence of diets of dead migrations (Ciozzolo et al., 1994), the samples from all five depths were combined to analyse size-class frequencies of the whole population during each month.

The logarithmic-difference technique of Bhattacharya (1967) was used to calculate the mean and variance of each normally distributed component in the polymodal size distribution and to analyse the population size structure. A chi-square test was performed to verify the fitting of expected and observed monthly frequencies. The mean lengths of the nonmodal components were linked by months (Skellam, 1972) to obtain stepped survivorship curves; monthly growth increments were calculated by following each cohort in time. The point of sex reversal was identified by following the fate of each sex in a plot of life cycle.

A static life table was constructed to calculate survivorship and mortality (Begon & Mortimer, 1986), in terms of $Q_0$ (‘age-specific mortality rate’; Halldane, 1949) and $k_0$ (‘growth power’; Varley & Gradwell, 1970). No information is given on fecundity of single females, because there was evidence of loss of eggs during fixing and sorting of samples. Data on fecundity and recruitment were provided only by the population dynamic analyses. Correspondence analyses (Benzecri, 1980) was carried out to identify the main phases of the life cycle and to confirm results obtained by the logarithmic-difference technique.

To evaluate the influence of primary production on the life cycle, regressions of the monthly frequencies of egressive females and juveniles on the abundance of epiphytes were calculated. These analyses were carried out on data obtained within the same 12 months of the study (Matazolla & Buta, 1989).

The statistical methods used in this study were chosen to study the life cycle on the basis of the ecological data sampled; previous studies of the life cycle of this species were based mainly on the results of histological techniques.

3. Results

The assemblages in the replicates for each station were not significantly different ($p<0.05$, Wilcoxon tests). Cluster analysis also revealed levels of similarity of the replicates at most stations; therefore, the replicates of each station were pooled.

A total of 5930 individuals of H. bersei were analysed. Population size increased from August to September, with most individuals found from 1 to 10 m (Fig. 1). Although this species lives preferentially in shallow beds (Zarinpary-Alvarez, 1964), the percentage of juveniles and males collected at 10 and 15 m in May and June was slightly higher than in other months.

The abundance pattern for the whole population was obtained by summing the
Fig. 1. Depth occurrence of juveniles (a), males (b) and females (c) of H. immer at five sampling depths. Both sexes show an increase in September, in accordance with the abundance of juveniles.
number of individuals of each size class at each depth (Fig. 2). Sexes were pooled in this representation, in the attempt to identify main periods of recruitment on the basis of the shift of the modal classes to lower sizes. One size class was clearly prevalent from June to September, and the existence of several size classes is particularly evident in March, April and May. In September, the modal class was shifted from moderately large to small animals. The means of the normal components for each month, identified using the logarithmic difference technique, is shown in Table 1a.

The analysis of sexes indicated that individuals under 6.5 mm are sexually undifferentiated juveniles. Specimens measuring between 6.5 and 17 mm include both males and females, while individuals over 17 mm are exclusively female (Fig. 3). The lower size limit for sexual maturity in both sexes was 6.5 mm. Juveniles and males exhibit

![Graph showing size distribution and recruitment patterns](image-url)

Fig. 2. Monthly size frequency charts for the population of H. bosophilius (individuals pooled from 1 to 25 m³); bars represent the abundance of each size class (1 mm).
<table>
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<td>(a) Population</td>
<td>20 →</td>
<td>17 →</td>
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<td>9 →</td>
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<td>(b) Life cycle</td>
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(a) Numbers indicate normal components (size classes) identified by the logarithmic-difference technique; the arrows follow the monthly growth of each class according to the method of stopped seaweed consumption curve; empty cells after "" indicate that the age class is no longer present. Results demonstrated that the cycle is completed in ~18 months but some individuals can live more than 2 yr; (b) main results of the life cycle study. No. ind. = total number of individuals; No. ovigerous = number of ovigerous females in the whole sample.
unimodal trends in size classes; the logarithmic-difference technique suggests that the size-frequency distribution of the female population is trimodal.

Juveniles were well represented in September, with a secondary peak in June (Fig. 4a,b); intermediate sizes (both sexes) were abundant from July to November (Fig. 4c); females between 11 and 17 mm in length were abundant in summer and showed a dramatic decrease in January and February (Fig. 4d); males over 14 mm reached maximum abundance in March and April (Fig. 4c). All individuals longer than 17 mm were females (Fig. 4f). In these representations the data of all depths were pooled to avoid errors produced by the continuous re-mixing of the population along the water column caused by diel migrations. However, no significant differences were found among the size and sex distribution of individuals sampled at different depths.

The highest mortality (expressed as both $Q_6$ and $K_6$, calculated by means of a static life table) was observed from October to December (Fig. 5), after larval settlement, although survivorship decreased throughout most of the year, increasing only from June to August. The analysis of static life tables also indicated two important periods of recruitment (expressed by negative mortality) during spring (May–June, with the lowest $Q_6$) and autumn (September).

The highest number of ovigerous females was observed in August (Fig. 6), although the highest proportion of females that were ovigerous was reached in April. There was a significant correlation ($R = 0.7$) between the pattern of epiphyte production (Fig. 6) and percent of ovigerous females. Plant epiphytes were mainly distributed at shallow depths (3–10 m), in accordance with the shrimp depth distribution. There were two peaks in the female size classes: one peak of small (5–17 mm) females in September (Fig. 7a), and another of large (17–33 mm) females in March (Fig. 7b); these two peaks coincided with the two reproduction periods. According to the Kozeny-Carman–Soninoff
test performed on the size classes of females collected in fall (August/September) and spring (March/April), the null hypothesis (homogeneity of fall and spring female populations) can be rejected at $p < 0.01$. The mean size of females in September was 8.0 mm (SD ± 1.8), in April, was 23.7 mm (SD ± 3.7). The logarithmic-difference techniques revealed a size class of juveniles that developed into females. The small females with direct development were called "Beta" females, and the large females that derive (according to Veillet et al., 1963) from sexual inversion, "Alpha" females.

A life cycle plot (Fig. 8) was obtained from the data shown in Table 1a, b, by linking the normal components by months, according to highest probability (Skelhorn, 1972), obtaining stepped survivorship curves. Two main size classes are present in March ($P_2$, $P_3$), and two recruitment periods are recognizable, in April ($F_1$, $F_2$) and September ($F_1'$, $F_2'$). In March, spawning by males ($P_2'$, 19 mm) and females ($P'$, 14
Fig. 5. Survivorship (S, %) and mortality, expressed both by \( Q_m \) (mortality rate) and \( K_m \) (mortality power) indices. All indices were calculated by means of the "Kaplan-Meier" method, on the basis of the monthly abundance of individuals.

\( nm \) produces a third size class of 3 mm (F1) that appears in April. The 14 mm female size class (F2) reaches a length of about 18 mm and disappears almost completely by August, while the male class (Fm) continues to grow, changes sex and, in the next spring, these females reach a length of 22 mm. Males born in April (F1) grow rapidly, reaching an average size of 7 mm in August. This generation (F1) joins that of the males.

Fig. 6. Number of oviparous females and percentage of all females that were oviparous during 1 year. Individuals pooled from 1 to 25 mm. The general pattern of aphid abundance (clumps grouped by months) in the P. aeruginosa leaf strain (from Mateles & Buis, 1983) is represented by bars. Both population data and aphid data were sampled during the same year.
Fig. 7. Number and size of females during the two main recruitment periods (a) August–September, (b) March–April, along the whole transect. Small-sized females were defined "beta"; large-sized females were defined "alpha".

from the previous generation (**P**<sub>0</sub>), shifted to females during winter, and produces a filial generation (**P**<sub>1</sub>), whose grandfathers were also their mothers.

A time delay exists between the spawning (detected by the presence of ovigerous females) and the appearance of a new size class in the size class frequency plot. It is due to the difference between the release of larvae and their metamorphosis to juveniles. The mean growth of the species, throughout the year, is about 1 mm per month. Males are mature at the age of 6 months; females spawn at the age of 6 months ("beta" females) or 1 yr ("alpha" females). Sexual inversion takes place in individuals aged, on average, 1 yr. "Beta" females range from 6 to 10 mm (11–12 mm females derive normally from sexual inversion after one year). The cycle is completed in 18 months, but a few individuals live more than 2 yr. The growth rate is slightly higher (about 1.3 mm/month) in summer than in colder months (about 0.8 mm/month).
Fig. 8. Life cycle diagram of *C. montagui*. Spring generation is thin line, autumn generation is bold line. Arrows on the ordinate indicate the size limits for both sexes. Numbers on abscissa refer to year. The female arrow is divided in order to indicate "Alpha" (larger) and "Beta" (smaller) female size limits. $P_0$, $P_1$, $P_2$ = parental male and parental female generations, respectively. $F_1$, $F_2$ = female generations of spring reproduction of Year 1 and 2, respectively; $F_1'$, $F_2'$ = male generations of fall reproduction of Year 1 and 2, respectively.

Fig. 9. Correspondence analysis ordination plot of the size-frequency class matrix. Numbers indicate the size (millions of units). Arrows were added to show a possible ordination of the dispersion into the space defined by $F_1$ and $F_2$. In the factorial space defined by this analysis, the proximity of points indicates a possible relationship between them. Smaller sizes are initial in September (the main period of reproduction), larger sizes (represented only by large females, as demonstrated by Fig. 1) are linked to April. The opposition of April in all the other months indicates its secondary reproduction basis.
The ordination obtained by correspondence analysis performed on the matrix "size frequency classes-months" (Fig. 9), is in accordance with the results presented in Fig. 8. Two main recruitment periods are opposed in the first (April) and second (September) quadrants and are linked, respectively, to the two types of females. The main recruitment period is in September, containing the 1–4 mm size classes (juveniles). The period "March–April-May" is dispersed in the first quadrant, linking the higher size classes opposed to September (main recruitment period) and to the winter growth period. Therefore, the plot is cyclic, and the dispersion of moult and size classes is in accordance with the results of the logarithmic difference analysis.

4. Discussion

The data demonstrate three age classes of H. incurvis, which overlap during most of the year and the previously undescribed development of small females from juveniles. The life cycle may be longer than 2 yr and sexual inversion does not take place in some individuals. This contrasts with a 2-year cycle proposed for this species by Veillet et al. (1963). The direct development of some females, in a species characterised by protracted hermaphroditism, however, has also been observed in other species of decapods. Allen (1959) observed that increased growth rate, rather than the attainment of a certain size, was related to sex reversal in Pandalus borealis, in this species both primary and sex-reversed females were found. Temperature was found to play a considerable role in sex reversal (Allen, 1959).

The strategy of sexual inversion observed in this study is consistent with the two periods of reproduction. It produces in September, at the time of the main recruitment, a maximum abundance of both sexes. The two periods of reproduction, in their turn, coincide with the optimal temperature for larval development (21°C; Regnaut, 1969a).

The first summer growth, probably due to high temperature and abundance of food, explains the juvenile peak evident only in September. In fact, sexual differentiation of the April generation occurs in August. In winter, fathers of the first generation (FL; Fig. 8) develop into females, while the younger generation (FL) produces males, that in turn reproduce in the following March. In September, females are larger than males of the same generation and maintain this difference throughout their life, producing a parallel generation, slightly larger than FL.

According to this model, high winter mortality (due to scarcity of food or higher predation pressure in the low canopy) dramatically reduces the percentage of females (older individuals) in the population. In fact, only 7% of individuals recruited in September were present in April (17 months after recruitment). The population, which is a main link in the chain between the grazers and large carnivores in the P. oceanica food web (Zupo & Frini, 1983), is stressed by predation from serranid, spard, labrid and scorpionid fishes (Bell & Hartemín-Vivien, 1983; Hartemín-Vivien & France, 1992; Zupo, 1993).

The number of females is maintained at a relatively constant level, because not all the specimens born in April develop as males. In fact, small directly developed females
(F1-F2; 7–8 mm) were detected, which can contribute to the adult female stock in September. Thus, the September reproduction is assured and produces a sufficient number of individuals for the next April recruitment. The main reproductive event in September, preceded by moom spawning activity in March, could be an adaptive strategy to overcome predation pressure by fishes and to provide sufficient numbers of “beta” females for the main mating period. In fact, the first reproductive period in March ensures an adequate number of parental individuals at a relatively low energy cost. These relationships are supported by the observation of two annual peaks of recruitment, two annual peaks of mortality and of two types of ovigerous females (one small which does not pass through a male stage) sampled in March-April and August-September, respectively.

The correspondence between the seasonal pattern of epiphyte production along the transect, the depth distribution of this species and the percentage of ovigerous females at each depth confirms the relationship between the life cycle of the shrimp and epiphytic food availability. This agrees with the concept of “sexual inversion due to geometric components expressed a different levels of development energy” (Reverberi, 1950). Moreover, Regnaux (1960) found a microphagous (planktrotrophic) nutritional pattern for H. borealis larvae, and the monthly pattern of juvenile abundance coincides with that for phytoplankton (Scotto di Carlo et al., 1985). Therefore, the present study suggests that the abundance of phytoplankton affects the depth distribution of the species, and that the abundance of P. aeraria epiphytes influences the depth distribution and presence of ovigerous females.

When food for larvae is abundant (spring phytoplankton bloom and summer production of epiphytes), there is a small reproductive event involving large males and females, the latter having developed in winter, after sexual inversion. In September there is a massive reproductive event involving small males and both “beta” females (mainly derived from the previous reproductive period and directly developed) and “alpha” females (females that have passed through a male stage).

These conclusions are also supported by data on nourishment of larvae (Regnaux, 1968), preferred temperatures (Regnaux, 1969a) and the “energy levels” theory (Reverberi, 1950). In summer, when food is abundant in the meadows, some individuals develop directly as females, while individuals born in September find less abundant food and follow the normal growth curve, with a first phase as males (lose energy) and a second as females (high energy). Interestingly, the small number of larvae born in March-April exploit the high abundance of food found in summer, whereas the large reproductive burst in August-September has a lower food source (Mazzella & Bius, 1989).

Sexual inversion primed by different energy levels is also supported by Le Roux (1963), who defined three periods of larval development of H. borealis. The author observed poecilogyny in this species, with the presence of 5 or up to 10 zoeal stages and hypothesized that the “transitory stages” between the regular stage IV and the last stage before the postlarvae were a consequence of variable feeding type and abundance of food.

The high number of young individuals present in the meadows during the period of highest phytoplankton abundance, and the relationships among depth distribution
pattern of the species, the distribution pattern of oviparous females and abundance of epiphytes, show that the life-cycle of *H. immersa*, by means of its peculiar strategy of sexual inversion, is in synchrony with environmental factors (e.g. food and temperature) and the plant production cycle.

Acknowledgements

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References


INTRODUCTION

The shrimp Hippolyte inermis Leach lives in seagrass meadows (Zariguie Alvarez 1968, Guillen Nieto 1990, Gambi et al. 1992) and undergoes a process of protandric sex reversal (Yaldwyn 1966), well demonstrated by previous investigations (Reverberi 1950, Veillet et al. 1963). However, it has been demonstrated that not all individuals in natural populations undergo the sex reversal process. Small females, presumably derived from direct differentiation, were also found in Posidonia oceanica meadows (Zupo 1994). Two reproductive periods that are synchronised with the seasonal growth cycle of P. oceanica (Buia et al. 1992) were detected in natural populations of the shrimp (Zupo 1994). The first period occurs in spring with the appearance of both males and females; the second period occurs in fall, with the appearance of males which then undergo sex reversal during the next year, after the following spring reproduction (Veillet et al. 1963). Small (‘beta’) females exhibit maximum abundance in association with blooms of epiphytic diatoms (Mazzella & Ott 1984); large females (‘alpha’ females, derived from sex reversal) first develop as males in September, during the period of minimum field abundance of epiphytic diatoms.

Immature individuals of this opportunistic, herbivorous shrimp (Zupo & Fresi 1985) feed on the most common epiphytes of Posidonia oceanica leaves, predominantly on microalgae, macroalgae, dinoflagellates of the genus Prorocentrum and some animals (e.g. Bryozoans, Foraminiferids). Adult individuals feed on microalgae, macroalgae and a few animal organisms. This diet pattern is common to several species of natantian decapods (Regnault 1969c, Nelson 1981, Gutiérrez-Yurrita et al. 1998). Adult individuals feed on microalgae, macroalgae and a few animal organisms. This diet pattern is common to several species of natantian decapods (Regnault 1969c, Nelson 1981, Gutiérrez-Yurrita et al. 1998). The diet of beta females is significantly different from that of males in the field (Zupo & Mazzocchi 1998). The gut contents of these females are dominated by some species of benthic diatoms, e.g. Cocconeis spp. that are particularly abundant (Mazzella et al. 1989) in the period of their development (spring). In contrast, the gut contents of alpha females are characterised by a lower abundance of diatoms; Cocconeis spp. in particular, are less abundant in the field and in gut contents of alpha females (Zupo & Mazzocchi 1998) in the period of their development (autumn).
The observed dietary differences and the significant relationships of the shrimp life cycle (alternation of alpha and beta females) with the seasonal pattern of abundance of diatoms in the leaf stratum of *Posidonia oceanica* (Buia et al. 1992), suggested the hypothesis of an influence of microalgal food on the sex reversal mechanism of this species (Zupo 1994). A laboratory experiment was devised and carried out to test the hypothesis that diatoms of the genus *Cocconeis* may influence the sex reversal of *H. inermis* (Zupo 1994), promoting the development of small females directly differentiated.

**MATERIAL AND METHODS**

*Hippolyte inermis* specimens were collected in Lacco Ameno (Bay of Naples, Italy), in the same site where samples were previously obtained for the investigation of the shrimp life cycle (Zupo 1994). A 400 µm mesh net (diameter 50 cm) was towed horizontally above *Posidonia oceanica* leaves at depths between 5 and 15 m. Two sampling seasons, April and September 1997, were chosen for sampling and rearing experiments, corresponding to the reproductive peaks of this species. Each sample was quickly sorted on board the boat to identify *H. inermis* specimens. In the laboratory, the collected individuals were examined under a dissecting microscope to confirm their identification (Zariquiey-Alvarez 1968), and their total length (tip of the rostrum, to the posterior medial notch) was measured. Their sex was determined (Fernández-Munoz & García-Raso 1987, d’Udekem d’Akoz 1996), and specimens in good condition were divided into 20 couples.

Individual couples were reared in 1000 ml flasks, containing 800 ml of 0.25 µm filtered seawater, enriched with a culture of the rotifer *Brachionus plicatilis* (2 ind. ml⁻¹). Flasks were reared in a thermostatic chamber, at a fixed temperature of 18°C, with a photoperiod of 12 h and an irradiance of 400 µE m⁻² s⁻¹. Shrimp food (Tetra AZ300 Artificial plankton for shrimps Type 1; Tetra Werke, Germany; 0.5 g) was added daily to each flask. Flasks, protected by gauze plugs to limit evaporation, were gently aerated. The medium in each flask was filtered daily through a 0.25 µm mesh net and replaced with fresh seawater enriched with rotifers; nets were checked under a stereomicroscope for the presence of larvae.

**Culture of plant and animal items.** Culture dishes were maintained in the same thermostatic chamber hosting the experimental shrimps to obtain sufficient amounts of plant and animal items to be fed to larvae and postlarvae. Monoclonal cultures of benthic diatoms available in the 2 periods of shrimp reproduction (Cocconeis neothumensis var. marina, Nitzschia sp.) were isolated from *Posidonia oceanica* leaves (Mazzella & Buia 1989). Diatoms were reared in Petri dishes (50 mm diameter) at 18°C in a sterilised F2 medium (Sigma Chemical), and were transferred every 5 d. Monoclonal cultures of macroalgae (*Enteromorpha sp.*) were also isolated from *P. oceanica* leaves and were reared in Petri dishes (diameter 10 cm) at 18°C, in a sterilised Provasoli (1963) medium. In addition, monoclonal cultures of phytoplankton *Dunaliella* sp., *Chlamydomonas* sp. and *Isochrysis* sp. were reared in 500 ml flasks at 18°C, using a sterilised K (Keller et al. 1987) medium with transfers every 10 d. Monoclonal cultures of *Brachionus plicatilis* were reared in 500 ml flasks at 18°C, in 400 ml of 0.45 µm filtered seawater enriched with a phytoplankton diet (1/3 *Dunaliella* spp., 1/3 *Isochrysis* spp., 1/3 *Chlamydomonas* spp., at a final cell concentration of about 10⁵ cells ml⁻¹), transferred every 7 to 8 d. *Artemia salina* cysts (New Technology, Bryne Shrimp, NT Laboratories Ltd) were reared in prefiltered (0.45 µm) seawater at 28°C, and freshly hatched nauplii were fed to shrimp larvae.

**Experimental procedures. Larval phase:** Each feeding experiment consisted of a larval and a postlarval phase. The complete experimental plan called for 3 replicates for each of 4 postlarval feeding treatments (described below). Newly hatched larvae from each female used were counted and divided using a Pasteur pipette into 2 groups of 100 individuals, and immediately transferred to 1000 ml flasks containing 800 ml of filtered (0.45 µm) seawater. Five zoal stages were observed, as described by Le Roux (1963). In flasks used to rear larvae for 3 of the postlarval treatments, the seawater was enriched (Le Roux 1963, Regnault 1969a) with *Artemia salina* freshly hatched nauplii (1 ind. ml⁻¹), *Brachionus plicatilis* (3 ind. ml⁻¹), *Dunaliella* sp. (10 000 cells ml⁻¹), *Nitzschia* sp. (14 000 cells ml⁻¹) and compound shrimp food (Tetra AZ300 Type 000; Tetra Werke, Germany; 150 mg per 800 ml). In the set of flasks used to rear larvae for the fourth postlarval treatment (GA(C)), *Nitzschia* sp. was replaced by *Cocconeis neothumensis* during the larval phase (14 000 cells ml⁻¹) to check the influence of this diatom on the larval development (Fig. 1).

An experiment was started when 6 females produced at least 200 larvae each, to be divided into flasks for the 4 treatments (Fig. 1). The larvae produced by other females, in lower abundance, were discarded. In September, when the mean size of females was lower than in April, only 2 replicates of the experiment were performed, as it was impossible to obtain, contemporaneously, 6 females producing at least 200 larvae each. The September experiment was therefore conducted on 800 larvae produced by 4 females (Replicates 1 and 2, Fig. 1).
Water in each flask was aerated by means of an airstone (Rice & Williamson 1970). Flasks for larval culture were reared in the same thermostatic chamber, on the same surface where adult individuals were reared, at 18°C and were protected by gauze plugs. The water temperature and the other abiotic factors (irradiance, photoperiod, salinity, pH, oxygen and ammonium concentration) were maintained constant during both periods of experiment. All experimental flasks were placed in a single row, at the same level in the chambers, to guarantee identical experimental conditions for all the replicates. Every 2 d, larvae were gently filtered through a net (0.25 µm mesh), counted and transferred to new flasks with identical fresh culture media. At the same time interval, 2 individuals were collected and fixed in 4% glutaraldehyde, to be measured using an electronic system of image analysis (Image Pro Plus, Media Cybernetics).

The number of larvae decreased during the experimental period, due both to natural mortality and the sampling of individuals at 2 d intervals. The larval growth phase lasted about 25 d and was checked every 2 d, to confirm the settlement of all individuals as completely developed postlarvae.

**Postlarval phase:** The postlarval phase of the feeding experiment started when all larvae settled. The 4 diet treatments for the postlarvae were (1) GADF: green alga Enteromorpha sp. + dry food (Tetra AZ300 Artificial plankton for shrimps Type 000); (2) DF: dry food (Tetra AZ300 Artificial plankton for shrimps Type 000); (3) DFC: Cocconeis neothumensis + dry food (Tetra AZ300 Artificial plankton for shrimps Type 000); and (4) GA(C): Postlarvae fed on *C. neothumensis* during the larval development phase were then fed on green alga Enteromorpha sp.

The 4 treatments, for each of 3 replicates, were obtained by larvae produced by 2 females of *Hippolyte inermis*, following a semi-randomised experimental plan (Fig. 1). At the start of the postlarval experimental phase, surviving individuals in each flask were reduced to 40, or higher (to avoid any influence on development due to density), and they were transferred to 1000 ml flasks containing 800 ml of 0.45 µm filtered seawater and the selected food items. At 2 d intervals, postlarvae were collected by a 0.45 µm net, counted, measured using a millimeter scale placed under a Petri dish, checked for their general conditions, and transferred to new flasks with the addition of clean water and food items, consistent with their diet treatment. This phase ended after about 45 d from hatching, when postlarvae achieved sexual maturation, at about 5 to 6 mm total length.

**Analysis of sex.** Sex and size were checked on narcotised shrimps under a binocular microscope, and identified by observing the shape and size of the first 2 pleopods. For this purpose, all shrimps were tested twice during the experiment, i.e. after 35 and 45 d from hatching. Narcotisation was devised (Smaldon & Lee 1979) to avoid damage to the experimental shrimps. Each individual was immersed for about 30 s in a well-mixed solution of 0.1 µl chloroform in 250 ml of seawater. Narcotised shrimps were easily manipulated, so that the ventral side was visible, and their sexual maturation was assessed under a dissecting microscope (40x). After sexual identification, shrimps were quickly transferred to a Petri dish with fresh seawater, where they recovered completely after a period of 30 to 90 s.

After 45 d, when most individuals had reached sexual maturity, all shrimps were preserved in 70% alcohol. Their second pleopods were excised under a binocular microscope and mounted on a slide for final determination of sex.

**Statistical analyses.** Linear regressions were applied to the shrimp size data, collected every 2 d throughout the experiment periods of both larval and postlarval growth. A growth curve was calculated for each treatment. The slopes obtained for each treatment were compared by ANCOVA (Sokal & Rohlf 1995) to examine any difference in the growth of shrimps under different diet regimes.

To test whether the appearance of beta females was influenced by the diet, a McNemar’s test (Sokal & Rohlf 1995) was applied to the percentage of males and females in the different treatments, for each replicate. For this purpose, data obtained in the 3 postlarval treatments without diatoms
(GADF, DF, GA(C)) were compared to the data obtained in the treatment with the presence of Cocconeis neothumensis during the postlarval growth (DFC). Similarly, the data obtained in the 2 postlarval treatments without diatoms (GADF, DF) were compared to the data obtained in the treatment with the presence of C. neothumensis during the larval growth (GA(C)).

RESULTS

During the 2 field sampling periods (April and September), 71 and 57 adult individuals were collected, respectively. The size differences between individuals collected in April (females, average 21.00 mm; males, 10.12 mm) and in September (females, 17.80 mm; males, 10.61 mm) were not significant (t-test; p = 0.351). The size of females used for larval production was between 20 and 26 mm in April and between 14 and 20 mm in September. Therefore, all females chosen for larval production were alpha females. The size of males used for reproduction was between 8 and 12 mm in both April and September. The experimental system devised for reproduction and feeding of larvae was sufficient to obtain, on the whole, 3160 larvae, 1200 of which were used for feeding experiments in April and 800 in September. No aggressivity was observed between the 2 adult individuals reared in each flask. The colour of reared individuals, however, changed during the experiment, from greenish to whitish, according to the mimicry characterising this species (Bedini et al. 1997).

The production of larvae coincided consistently with the moult of females. The number of larvae produced by each female was highly variable, depending on both the season and the size of females. The maximum production was observed in April (237 larvae from a single female), the minimum in September (32). Mortality during the larval phase did not exceed 32% in all treatments, and during the postlarval phase was 49%, on average (Table 1). The most critical period was represented by sex determination at Day 35, as not all narcotised individuals recovered. The p-value for the treatment by the covariate interaction (ANCOVA) was 0.820, so the assumption of homogeneity of slopes is plausible (Table 2), although a slightly faster growth was obtained with the diet DFC (slope 0.15), while the slowest growth was obtained with the diet GA(C) (slope 0.10). The growth process was almost continuous, but larvae from all treatments exhibited a period of lower increase in length from Days 15 to 22. Length increases became greater after the settlement to postlarvae. On average, Hippolyte inermis exhibited an increase in length of 0.186 mm day\(^{-1}\) until Day 35 of the experiment. The maximum size, Day 45 of the experiment, was 10 mm total length, reached by postlarvae under GADF, GA(C) (April; Fig. 2) and DF (both April and September; Figs. 2 & 3) treatments.

The differences between sex ratio and size distributions obtained in April and September experiments, in each treatment, were not significant (Figs. 2 & 3; t-test, p > 0.05), although the total number of adult individuals obtained at the end of the experiments in September...

Table 1. Results of the feeding experiments at Day 45. Total number of males (M), females (F) and juveniles (J) is given for each replicate, treatment, and experimental period. Percent mortality was calculated from the start of postlarval treatments. Abbreviations of the 4 dietetic regimes are explained in the text.

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Table 2. R\(^2\) and slopes of the growth curves obtained by linear regression of the size of individuals sampled within each treatment versus the time of experiment, until Day 45.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>R(^2)</th>
<th>Slope</th>
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<tr>
<td>GADF</td>
<td>0.966</td>
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<tr>
<td>DF</td>
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</tr>
<tr>
<td>DFC</td>
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<tr>
<td>GA(C)</td>
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<td>0.10</td>
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The number (158) was lower than in April (234) due to the availability of only 2 replicates. The GADF treatment yielded only 1 female (9 mm total length) in the 3 replicates of April (Fig. 2A) and 2 females, of the same size, in the September experiment (Fig. 3A). This treatment yielded mostly males of between 7 and 9 mm total length, with the presence of a few immature ones between 5 and 7 mm at Day 45. The DF treatment yielded 5 females between 7 and 10 mm in April and 2 females between 8 and 10 mm in September. In this treatment a higher number of large males (8 to 9 mm) was observed (Figs. 2B & 3B). The DFC treatment yielded the largest abundance of females, both in April (35 ind.) and in September (26 ind.), between 5 and 9 mm (Figs. 2C & 3C). In this treatment, only 5 males larger than 7 mm were found in September; all the other males were small. The GA(C) treatment yielded only 3 females in April and 1 small female (5 mm total length) in September (Figs. 2D & 3D).

Striking differences between the DFC treatment and all the other treatments were observed by Day 35 of the experiments (Fig. 4). In both April and September the number of females at Day 35 was higher in the DFC treatment than the number of males and juveniles (Figs. 4A,C). The number of juveniles decreased considerably from Days 35 to 45 of the experiments, as most individuals reached sexual development (Fig. 4). The immature shrimps represented about 35% of individuals at Day 35 of the experiment, and decreased to about 10% of the total individuals at Day 45. There was a significant difference (McNemar’s test; p < 0.001) in the proportion of individuals between the postlarval treatment containing *Cocconeis neothu-\textit{menis}* (DFC) and all the other treatments (Table 1). Differences were significant (p < 0.001) for both experimental periods (April and September). No significant differences were detected between the results of the same treatment, when April and September data were
compared. Pooling data from all the replicates, individuals on diets without *C. neothumensis* comprised 85.39% males, 5.06% females and 9.55% juveniles (Fig. 5A). Individuals on the diet containing *C. neothumensis* in the postlarval period comprised 30.36% males, 60.71% females and 8.93% juveniles (Fig. 5B). No significant differences were found between the treatment containing only *C. neothumensis* in the larval phase (GA(C)) and the 2 treatments containing no diatoms in the postlarval phase (GADF, DF).

**DISCUSSION**

Larval diets containing both plant and animal items (Regnault 1969a) resulted in a sufficient number of postlarvae for feeding experiments. In the laboratory, larvae reached sexual maturity on average 45 d after hatching at a mean size of 7 mm. In contrast, field data indicated that shrimps may reach sexual maturity at 5 mm, about 30 d after hatching (Zupo 1994). These differences may be explained by the higher variety of food items available in the field. Diet may also influence the succession of larval stages and development (Le Roux 1963), and the results of the present investigation confirm the importance of animal items in the diet of larvae (Provenzano 1967, Amat et al. 1987). Both the size distribution and the sex ratio of individuals of each treatment exhibited no significant differences between the experiments of April and September, thus demonstrating that, in consistent environmental conditions, the females sampled in the 2 periods produced a generation of similar characteristics.

The results indicated that sex reversal in *Hippolyte inermis* is influenced by ingested food. As demonstrated by the GA(C) treatment, food in the larval phase had no effect on sex differentiation, as no significant differences were observed between the treatment containing *Cocconeis neothumensis* during the larval phase and the other treatments not containing this diatom. In contrast, the results of the DFC treatment demonstrated a clear effect of diatoms consumed during the postlarval growth period, from Days 25 to 45 of the experiment. The abundance of females in the treatments not containing *C. neothumensis* during the postlarval growth period (GADF, DF and GA(C)) was as low as 5%. This finding may explain the absence of beta females in the field in the September recruitment period (Zupo 1994), when the diatom is less abundant in the leaf canopy of *Posidonia oceanica*. The percent abundance of females in the treatment containing *C. neothumensis* (DFC) was 12 times higher than in all the other treatments, and may explain the abundance of beta females in the field during the April recruitment period, when diatoms of the genus *Cocconeis* reach their maximum abundance in the leaf canopy of *P. oceanica* (Mazzella & Ott 1984). These results are in accordance with the presence of *Cocconeis* in the gut contents of shrimps collected in the field in April (Zupo & Mazzocchi 1998).

The higher abundance of small females in the treatment containing *Cocconeis neothumensis* was clear by Day 35, even though 35% of individuals were still in the juvenile phase (Fig. 4). It is thus unlikely that females observed at the end of the experiment had passed through a short male stage. Most individuals reached their sexual maturity before Day 35 from hatching, and all but 10% completed their sexual development between Days 35 and 45 after hatching. Moreover, females produced by the DFC treatment exhibited a small size (7.3 mm, on average), compara-
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Although growth was almost constant over the experimental period, as demonstrated by the good fit of linear regressions, larvae exhibited a short period of lower growth between Days 10 and 20 of the experiments. This may be due to the passage through complex developmental stages and to a requirement for selected food items (Le Roux 1963). The presence of *Cocconeis neothumensis* in the postlarval diet produced a good growth of shrimps. In fact, shrimps under the DFC treatment exhibited a larger mean size at Day 45 than shrimps fed the DF diet. The average size of females in the DFC treatment, however, was smaller than the size of females in all the other treatments. Therefore, the development of a female gonad may require energies which slow the normal growth process (Dutz 1998).

No information is available on a similar effect of food on other benthic crustaceans, although protandric hermaphroditism is a common strategy in several species of decapod crustaceans (Yaldwyn 1966, Gherardi & Calloni 1993). It is well known that food may influence the hormone metabolism in several organisms, such as insects (Harrison 1990), copepods (Jónasdóttir 1994), shrimps (Yano 1995) and fish (Bell et al. 1996). However, it is still unclear whether the effects of diatoms on the sex reversal of *Hippolyte inermis* may be due to an influence of dietary components on the hormone metabolism, or to a direct effect on the gonadic tissues (Charniaux-Cotton 1958). In fact, in this species, female gonadic buds are absent (Reverberi 1950), and the female gonad is built, in adult males, starting from undifferentiated cells. Therefore, a direct influence of food substances with hormone activity on the female gonad may be negated, when the shrimp is still undifferentiated. Moreover, a lack of factors allowing the normal development could be excluded, when we consider that the growth rate in culture (about 4 mm mo⁻¹, on average) was higher than the average growth rate observed in the field (1.3 mm mo⁻¹; Zupo 1994). In contrast, sex reversal may be influenced in decapods by parasitic castration (Baffoni 1947), temperature (Allen 1959) or by the attainment of a given size (Noel 1973). *H. inermis* is sensitive to several environmental cues, at least during larval development (Regnault 1969b), and environmental factors may influence sex change in several crustaceans (Fleminger 1985, Arechiga & Rodriguez-Sosa 1997) and affect the structure of phytoplankton communities (Edgar 1983, 1990). However, identical experimental conditions characterised all the tests performed. Le Roux (1963) also hypothesised that the ‘transitory stages’ between the larval stage IV and the last zoea before the postlarva were a consequence of type and abundance of food, but our data indicate that the influence of *Cocconeis neothumensis* on sex reversal is limited to the postlarval period of development.

Diatoms have been demonstrated to influence the ecology and the life cycle of other crustaceans (Miralto et al. 1995, Dutz 1998). Egg production and hatching success in individuals of the copepod *Centropages typicus* decreased when diatoms were fed to adult females (Ianora et al. 1995). Diatoms of the genus *Thalassiosira* are able to arrest the embryonic development of the copepod *Calanus helgolandicus* (Poulet et al. 1994). The production by several species of diatoms of compounds detrimental to the development and survival of grazers may have major implications on secondary production (Miralto et al. 1996). However, in *Hippolyte inermis*, other effects (e.g. destruction of the male gonadic bud, allowing a direct development as female, or direct influence on the androgenic gland) could be possible (Hoffman 1969, Adiyodi & Adiyodi 1970, Miralto et al. 1999), resulting from co-evolutionary processes. In fact, *H. inermis* is largely adapted to the life in *Posidonia oceanica* (d’Udekem d’Acoz 1996) and the pattern of abundance of alpha and beta females is synchronised with the seasonal abundance of micro- and

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**Fig. 5.** Percent of males, females and immature shrimps obtained within the postlarval feeding treatments (A) without (GADF, GA(C), DF) *Cocconeis neothumensis* diatoms and (B) with (DFC) *C. neothumensis*. Data from all the replicates in both the experimental periods (April and September) were pooled.
macroalgae in the field (Zupo 1994). Therefore, the toxic effect of diatoms may be translated in this species into a spring signal for the development of beta females, as observed in other crustaceans (Svensen & Tande 1999). It was demonstrated that the presence of beta females is a crucial factor in maintaining a constant sex ratio, allowing the September reproductive burst (Zupo 1994). Therefore, the presence of beta females in the spring population, triggered by the feeding on diatoms abundant in this season, may be an adaptive strategy (Bull 1987) to provide a sufficient number of females for the next mating period in September.

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LITERATURE CITED


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Influence of diet on sex differentiation of *Hippolyte inermis* Leach (Decapoda: Natantia) in the field

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Key words: *Hippolyte inermis*, shrimp, food, sex reversal, development, *Posidonia oceanica*

Abstract
The gut contents of the shrimp *Hippolyte inermis* were investigated for 1 year along a depth transect through a seagrass bed. Besides size, sex and weight of all individuals were recorded. The diets of immature and adult individuals were compared to detect any influence of food on sex development, since previous investigations indicated a correlation of the life cycle of this protandric species with the abundance of algal food in the environment, and laboratory experiments demonstrated the effect of diatoms of the genus *Cocconeis* on the direct development of females. Results indicated that the shrimp is an opportunistic herbivore, able to feed on both plant and animal items, with a preference for macroalgae and diatoms present on the leaves of *Posidonia oceanica*. Small females, deriving from direct differentiation, had a diet significantly different from that of males. The difference was due to a larger abundance of microalgae in the guts of young females. The influence of microalgal food on the sex reversal mechanism of this species, previously detected through laboratory experiments, was demonstrated to control the life cycle of *H. inermis* in the field.

Introduction

*Hippolyte inermis* Leach, 1815 is a small shrimp (maximum length is 25 mm) living in shallow waters of the Mediterranean Sea and along the Atlantic coast of Spain (Zariquiey Alvarez, 1968). It forms stable populations in seagrass meadows (Gambi et al., 1992), mainly in *Posidonia oceanica* and *Cystoseira nodosa*, but it is also present in other coastal environments (Guillen Nieto, 1990). Most individuals exhibit a green mimic colour, although they can shift to grey, brown or white, according to the environmental conditions (Bedini et al., 1997).

Investigations by Reverberi (1950) and Veillet et al. (1963) demonstrated individuals experiencing a male stage prior to switching to female (i.e. protandric sex reversal; Gherardi & Calloni, 1993). It is also well known (Le Roux, 1963; Regnault, 1969a) that juvenile diet is based on microalgae, but there is a lack of information about the feeding ecology of postlarvae in the field. Sex differentiation occurs at a size of 5–7 mm of total length (Veillet et al., 1963); sex reversal is observed in individuals of 10–13 mm, corresponding to the age of 7–12 months. Not all individuals exhibit sex reversal; in fact, young females of 5–6 mm size were present in natural populations (Zupo, 1994). They are smaller than any male and supposedly derive from direct differentiation. Large females, deriving from sexual inversion, were designated as alpha females, while small females, directly developed, were designated as beta females (Zupo, 1994).

Two main periods of recruitment, in spring and fall, were detected in the life cycle of *H. inermis*. Individuals born in spring grow quickly and develop as both females or males, while individuals born in fall grow slowly and develop as males, reverting sex in the next spring. A significant relationship between epiphyte abundance in *Posidonia oceanica* meadows and the frequency of ovigerous females was demonstrated (Zupo, 1994). Moreover, the period of maximum abundance of beta females in natural populations corresponds to a massive epiphytic production in the leaf stratum of *P. oceanica* (Mazzella & Buia, 1989). Laboratory experiments (Zupo, 2000) demonstrated that diatoms of the genus *Cocconeis* allow the direct development of beta females. Therefore, mi-...
croalgal food influences the life cycle of *H. inermis* in the laboratory and it may play a role, also in the field, in the sex differentiation of this species. Therefore, a field investigation was devised to study the relationships existing between the diet of shrimps during the phases of sex development and the pattern of abundance of *alpha* and *beta* females.

#### Materials and methods

Two replicate samples of *Hippolyte inermis* were monthly collected, during 1 year, by a hand-towed net (0.4 mm mesh size) in Lacco Ameno d’Ischia (Gulf of Naples, Italy) along a depth transect at 1, 3, 10, 15 and 25 m depth (Fig. 1). This method (Ledoyer, 1962; Russo & Vinci, 1991; Gambi et al., 1992) is considered to be best suited to collect vagile organisms associated with the leaf stratum. It is semi-quantitative and allows to collect a large number of individuals to submit to further analyses. After collection, animals were deep frozen to prevent digestion and successively fixed in 70% alcohol (Zupo & Fresi, 1985). After identification, the total length of all individuals (except damaged specimens in which total length or sex could not be determined) and their sex were recorded. The total length was measured from the tip of the rostrum to the posterior medial notch of the telson, pressing each specimen against a metal ruler, to obtain a measure of bodies at the maximum extension. Sex was determined based on the form and the presence of setae on pleopod II (Fernandez-Muñoz & Garcia-Raso, 1987). Intra- and inter-sample similarity was evaluated by performing a mean linkage cluster analysis on the central scalar product matrix obtained from the ‘size vs. stations’ matrix (Orloci, 1978).

All shrimps belonging to the ‘critical’ sizes for the study of sex reversal (Zupo, 1994) were selected: 3 mm (all juveniles); 7 mm (juveniles, males and females); 17 mm (all females). The selected individuals were dissected and their guts were analysed using an optical microscope, to identify all prey up to the lowest taxonomic level. The abundance of each food item was evaluated using an ocular reticule. The gut contents were homogeneously dispersed on a microscope slide and the percentage of squares containing at least one fragment of each item was recorded. A matrix ‘individuals vs. gut contents’ was obtained and analysed.
Figure 2. Percent abundance of food items found in the gut contents of juveniles, females and males, after pooling all yearly data.

Results

Cluster analysis revealed high levels of similarity of the replicates for each station; therefore the replicates of each station, for each month, were pooled. In total, 213 individuals (60 immatures, 76 males, 77 females) belonging to the ‘critical’ sizes for the study of sex reversal were selected, and their gut contents were analysed. The analysis of gut contents revealed a similar pattern in the abundance of food items among the diet of males, females and immature individuals (Fig. 2). Kruskal-Wallis test indicated no significant differences among the gut content assemblages observed at different depths or in individuals of different size. Also the differences among the diets of sexually immature individuals, males and females were not significant. Diatoms of the genus Licmophora and sponge tissues, however, were present only in the diet of adult individuals (Fig. 2).

Microalgae were consumed by all individuals, but immature shrimps fed preferentially on some dinoflagellates, such as Prorocentrum. Erect and encrusting algae represented common and abundant items for all the individuals, although animal prey, represented by sessile species living on the seagrass leaves or small animals living in the epiphytic layer, were present in most guts. When the total abundance of all items was considered (Fig. 2), macroalgae and diatoms of the genus Cocconeis (two common items in the leaf stratum) were the most consumed items, followed by animal prey (foraminiferans), and other micro-organisms. Sponges and such diatoms as Licmophora and Achnanthes were the least preferred items. Prey easy to detach, such as erect algae and foraminiferans, were preferred to motile prey, such as small crustaceans (mainly copepods).

The abundance of microalgae in the guts was higher at shallowest depth (1 m), where a higher abundance of dinoflagellates of the genus Prorocentrum and diatoms of the genus Amphora were found (Fig. 3a). The highest abundance of macroalgae was found in guts collected at 10 m depth (Fig. 3b), coinciding with a decrease in the gut abundance of microalgae. Erect algae were constantly preferred to encrusting algae. Animal prey, mainly represented by foraminiferans and bryozoans, were consumed at all depths (Fig. 3c).

Taking into account the seasonal trend of the appearance of each item in the guts (Fig. 4), diatoms of the genus Cocconeis were consistently the most common microalgal prey, with maximum abundance in April–May, the period of higher occurrence of beta females. Macroalgae followed a trend similar to that of the plant life cycle (Fig. 4b); however, erect algae were consistently preferred to crustose ones. Among animal prey, foraminiferans were the preferred item in several months, although bryozoans were abundant in the gut contents all over the year (Fig. 4c). Crusta-
Figure 3. Percent abundance of food items found in the gut contents, along the depth transect, separated in the main food categories: (a) microalgae; (b) macroalgae; (c) animal prey. Data were pooled for all individuals sampled.

Copepods were particularly abundant in summer and fall, coinciding with the reproductive burst of some species of harpacticoid copepods. Chi-square tests performed on proportion of microalgae vs. other prey indicated that in April and May the proportion of microalgae in the diet was significantly different ($p<0.001$) from all the other months (Figs 5a, b). Similar tests, performed for all other months, indicated no significant differences. Also the differences between the proportions of...
microalgae and other prey, performed among different depths, were not significant.

A correspondence analysis performed on the matrix individuals vs. food items indicated two important poles (Fig. 6), defined by the first two factors. In the first quadrant, the carnivore pole, identified by all animal prey; in the second quadrant, the diatom pole; macrophytes were ordered centrally and linked to small males. Small females (beta females) were linked to the diatom pole, while large females (alpha females) were linked to the animal pole. Immatures were ordered in the third and fourth quadrant, dragged down by the item "Prorocentrum" and linked to diatoms such as Tabellaria. Diatoms of the genus Coc-
Figure 5. Percent abundance of microalgae vs. all other items in the gut contents of individuals sampled in the period of development of beta females (a) and in all other months of sampling (b), compared to the results obtained in the laboratory (45 days after hatching), in a previous investigation (from Zupo, 2000) in which H. inermis was fed with diets not containing diatoms (c) and with the addition of diatoms of the genus Cocconeis (d).

cocconeis and Tabellaria were ordered centrally among immature individuals (J3) and young shrimps at the age of differentiation belonging to both sexes (F7, M7), although young males were closer to macroalgae and young females were closer to the diatom pole. A correspondence analysis performed on the matrix gut contents vs. depth confirmed the importance of Cocconeis and macroalgae, consumed at all depths. Whenever possible, plant materials were the preferred prey. The analysis, however, showed that the abundance of food items in the guts was also in accordance to the depth distribution of prey in the field: the abundance of some animal prey in the guts increased with depth, in accordance with decrease of plant prey.

Results of cluster analysis performed on the matrix individuals vs. food items indicated three main groups of observations (Fig. 7). The first group contained only large females (17 mm), separated at a high level of variance. The second group was composed of individuals of 3 and 7 mm, which were further split into two clusters. The first sub-cluster contained juveniles and small females; the second contained juveniles and males.

There was a significant relationship between size of individuals and weight of their guts ($r=0.98$). Dry weight of guts, in the period of sex maturation, ranged from 0.030 mg ±0.01 (males), to 0.035 mg ±0.01 (juveniles) to 0.045 mg ±0.02 (females). Females exhibited the largest variability in gut weight, reaching 0.1 mg ± 0.04 at a size of 17 mm. The smallest juveniles (size 3 mm) exhibited the lightest guts (0.02 mg ± 0.01). No significant differences were detected among
Figure 6. Results of the Correspondence Analysis performed on the matrix ‘individuals vs. gut contents’. Observation and variable points are represented in the space defined by the first two significant axes, F1 and F2. Individuals of different size and sex are represented in bold (J3= juveniles of 3 mm size; J7=juveniles of 7 mm size; M7= males of 7 mm size; F7= females of 7 mm size; F17= females of 17 mm size). Acronyms refer to food items: Lic= Licmophora; Amp= Amphora; Ach= Achnanthes; Fra= Fragilaria; Coc= Cocconeis; Tab= Tabellaria; Pro= Prorocentrum; Oth= Other diatoms; Ere= Erect algae; Enc= Encrusting algae; For= Foraminifera; Cru= Crustaceans; Bry= Bryozoa; Spo= Sponges. The three main diet poles in the plot are indicated in italics.

Figure 7. Results of the cluster analysis performed on the matrix ‘individuals vs. gut contents’. Individuals are grouped on the basis of their gut contents. 3 mm, 7 mm and 17 mm indicate groups of individuals with total length of 3, 7 and 17 mm, respectively. M= males; F= females; J= immatures.
the gut weights of females, males and juveniles of the same size.

Discussion

The data obtained establish that *Hippolyte inermis* feeds on both plant and animal items. Besides slight differences in the diet between adults and juveniles or individuals of recent maturation, the patterns of abundance of food items in the guts appear consistent, when all yearly data are pooled. Erect and encrusting algae are an important dietary component and they are present, at the same abundance, in guts of individuals of different ages. A slight difference in the consumption of macroalgae was observed along the depth transect. The trend coincides with the pattern of abundance of erect and encrusting algae in the shallow meadow (Mazzella & Ott, 1984). An increase in the consumption of erect macroalgae was observed at the deep meadow (25 m), where a lower abundance of this item was recorded in the field (Mazzella & Ott, 1984).

This finding may be explained observing, at the same depth, a higher consumption of animal prey (mainly bryozoans and foraminiferans) and a lower presence of diatoms in the gut contents: at higher depths, *H. inermis* integrates the lack of microalgae in the field (Buia et al., 1992) with a higher consumption of macroalgae and animals. It is notable that the abundance of macroalgae and animal prey increased consistently with the decrease in the abundance of microalgae. Microalgae appear to be the preferred item, but they may be replaced by other prey when their abundance in the field decreases.

Due to this opportunistic strategy, the gut weight of individuals collected at different depths is consistent, according to size. In fact, the amount of food in the guts is not related with sex changes nor depth, as it was demonstrated that no significant difference exists among the different experimental groups. The amount of food ingested is only related to the size of shrimps among the different experimental groups. The amount of food in the guts is not related with sex changes nor depth, as it was demonstrated that no significant difference exists among the different experimental groups. The amount of food ingested is only related to the size of shrimps among the different experimental groups. The amount of food in the guts is not related with sex changes nor depth, as it was demonstrated that no significant difference exists among the different experimental groups. The amount of food ingested is only related to the size of shrimps among the different experimental groups. The amount of food in the guts is not related with sex changes nor depth, as it was demonstrated that no significant difference exists among the different experimental groups. The amount of food ingested is only related to the size of shrimps among the different experimental groups. The amount of food in the guts is not related with sex changes nor depth, as it was demonstrated that no significant difference exists among the different experimental groups. The amount of food ingested is only related to the size of shrimps among the different experimental groups.
Achnanthes, Fragilaria, Cocconeis), that trigger the direct development of beta females.

Diatoms have been demonstrated to influence the ecology and the life cycle of other crustaceans (Miralto et al., 1995). Egg production and hatching success in individuals of the copepod Centropages typicus decreased when diatoms were fed to adult females (Ianora et al., 1995). Moreover, the harmful impact of a diatom (Thalassiosira rotula) on the reproductive biology of the copepod Calanus helgolandicus was demonstrated (Poulet et al., 1994). The production, by several species of diatoms, of compounds detrimental to the development and survival of grazers, may have major implications on secondary production (Miralto et al., 1996). However, in this species, other effects could be possible (Adiyodi & Adiyodi, 1970), according to co-evolutionary processes (e.g. apoptotic disruption of the male gonadic bud, allowing a direct development as female). In fact, H. inermis is largely adapted to the life in P. oceanica (d’Udekem d’Acoz, 1996) and the toxic effect of diatoms could be translated, in this species, in a spring signal for the development of beta females. It was demonstrated that the presence of beta females is a crucial factor in maintaining a constant sex ratio, allowing the September reproductive burst (Zupo, 1994). Therefore, the presence of beta females in the spring population, triggered by the feeding on species of diatoms abundant in this season, may be an adaptive strategy to provide a sufficient number of females for the next mating period in September.

The results of the present investigation reinforce previous findings, suggesting a seasonal correlation of the life cycle of Hippolyte inermis with the pattern of abundance of epiphytic algae in Posidonia oceanica meadows. They are in accordance with results obtained in the laboratory (Zupo, 2000), indicating a direct effect of diatoms on the development of beta females in this species. Maximum breeding intensity was observed in spring (Zupo, 1994), when the abundance of epiphytes in the leaf stratum is high (Mazzella et al., 1989). In the same season the largest burst of beta females was found and data of the present investigation confirm that some microalgae, very abundant in the field, are actively selected in the diet of young shrimps and correlated to the production of beta females undergoing a mechanism of direct development.

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Do benthic and planktonic diatoms produce equivalent effects in crustaceans?

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Do benthic and planktonic diatoms produce equivalent effects in crustaceans?

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Abstract

Hippolyte inermis Leach 1814 is a benthic shrimp characterized by a peculiar mechanism of sex reversal influenced by diatom foods. In fact, the appearance of primary females in spring is due to an apoptotic early disruption of the androgenic gland and of the male gonad, triggered by still unknown compounds present in diatoms of the genus Cocconeis. The influence of diatoms on the reproductive ecology and life cycle of planktonic crustaceans has been demonstrated previously: some planktonic diatoms produce aldehydes inducing apoptosis in the embryos and in the larvae of marine copepods, reducing their viability. Both benthic and planktonic diatoms therefore produce compounds having an apoptotic effect on some tissues of target crustaceans, although the ecological significance of the two processes is different: deleterious for copepod populations, regulative for shrimps associated with Posidonia oceanica. In the present article we experimentally administered specific planktonic diatoms, their fractions and compounds known to induce apoptosis in planktonic copepods, to H. inermis postlarvae, to check whether the apoptotic effect is due to an identical family of diatom compounds, and to establish whether the processes observed in the plankton and in the benthos, respectively, are analogous or homologous, from an ecological point of view. Our results indicated that diatom compounds acting in the two systems are different, since both planktonic diatoms and their aldehydes had negligible effects on the sex ratios of cultured shrimps.

Keywords: Hippolyte inermis, shrimp, sex reversal, diatom, apoptosis

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Introduction

_Hippolyte inermis_ Leach is a benthic shrimp (Zariquiey Alvarez 1968; d’Udekem d’Acoz 1996) well-known for its mimicry (Bedini et al. 1997) and for its peculiar strategy of sex reversal. The species is characterized by two yearly periods of reproduction, in spring and fall (Buia et al. 2000). Individuals born in fall develop as males and, after about 1 year, they shift their sex to female (Veillet et al. 1963), as observed in several other decapods (Yaldwin 1966; Adyodi and Adyodi 1970; Bauer 2000). Individuals born in spring, in contrast, develop both as males and females (Zupo 1994). They grow fast and mate the following September, to prime the fall reproductive burst. The different fate of populations born in spring and fall is apparently due to the diatom food available in the field (Mazzella and Buia 1989). Individuals born in spring were observed to ingest high biomasses of diatoms of the genus _Cocconeis_, while these microalgae are almost absent in the diet of the individuals born in fall (Zupo 2001). Laboratory experiments confirmed this field-based evidence (Zupo 2000). It was demonstrated that individuals born of the same females develop mainly as males or females according to the absence or presence of _Cocconeis neothumensis_ in their diet, respectively (De Stefano et al. 2000).

Reverberi (1950) observed a peculiar mechanism of sex reversal in this species. He demonstrated that the female gonad is produced from undifferentiated tissues only after the complete disruption of the testes. The absence of an ovotestis was recently confirmed also by Cobos et al. (2005). The sex change mechanism is therefore different from that observed in other decapod crustaceans (Charniaux-Cotton 1960; Charniaux-Cotton and Payen 1988; Calado et al. 2005), in which an ovotestis is normally observed and the development of an ovary follows the classical steps of a hermaphroditic sex change (both simultaneous or not; Bauer 2000). For this reason Reverberi (1950) stated that _H. inermis_ reverts its sex, but it cannot be considered hermaphroditic, because there is never contemporaneous presence of gonads or even of gonadic buds (Ginsburger-Vogel and Charniaux-Cotton 1982). This led to confusion in recent times because Cobos et al. (2005) discounted the possibility of observing sex reversal in the absence of an ovotestis, and the question is still under debate. However, Zupo and Messina (2007) demonstrated that the early disruption of the male gonad, preceding the development of an ovary, is due to apoptosis, i.e., programmed cell death (Raff 1998; Evan and Littlewood 1998; Vaux and Korsmeyer 1999) triggered by compounds present, in various concentrations, in different benthic diatoms. Zupo and Messina (2007) also demonstrated that the disruption of the androgenic gland and of the testis takes place in a very early postlarval stage: apoptosis of these organs was observed as early as 2 days after settlement, when active diatoms were administered in the diet.

The action of diatoms delivered with the diet is very specific and time limited. It takes place from the second to the 12th day of postlarval development and is targeted only against the androgenic gland (AG; Sagi 1988) and the male gonad (Zupo and Messina 2007). It is therefore a very fast and specific process and it leads to the complete disruption of the AG in the first days of postlarval growth and to the appearance of the female sex within a single moult cycle. In fact, the presence of active females (presence of an ovary) with external male appendages was observed before the moult (Reverberi 1950). Active males (presence of mature testis) with external female secondary characters are observed as well, when the moult precedes the disruption of the testis (Katakura 1989). Based on these interesting observations, an EU research project named Pharmapox was started in 2005. It is aimed at isolating, purifying, and characterizing the apoptotic compounds present.
in benthic diatoms and to determine their activity for biotechnological purposes (Hannun 1997).

The aim of the present study was to attempt a first extraction of benthic diatom compounds and compare their activity to that of planktonic diatoms. Miralto et al. (1999) demonstrated that diatoms may have an insidious effect on copepod reproduction. These authors also observed that the compounds present in some planktonic diatoms produce apoptosis both in the embryo and in the first larval stages, thus impacting both the survival and the viability of recruits (Romano et al. 2003; Ianora et al. 2004). This was supposed to be, however, a mechanism of defense useful for the diatoms, to reduce the impact of grazers during their plankton blooms (Miralto et al. 1996; Ianora et al. 2004). In contrast, the role of apoptosis in the benthic decapod *H. inermis* appears regulative for the species, since it leads to a higher fitness and guarantees a stable sex ratio in the population. The compounds produced by planktonic diatoms that are detrimental to the development of copepods are mainly aldehydes (Miralto et al. 1999). The compounds present in benthic diatoms, regulating the shrimp populations, are still unknown.

It is an important issue as to whether the same compounds induce apoptosis (with contrasting ecological effects) in benthos and plankton. In such a case, the activity of diatoms on crustacean populations in the two systems could be considered homologous from an ecological point of view. In contrast, the activity of diatoms in the two systems should be considered analogous if the apoptotic compounds they deliver to crustacean populations are different. We therefore performed extractions on both planktonic and benthic diatoms and administered dried diatoms and their extracts to postlarvae of *H. inermis*, to determine whether they produced similar effects on the proportions of females matured in each treatment.

**Material and methods**

We cultivated both planktonic and benthic diatoms. The planktonic diatoms (*Skeletonema costatum*) were cultivated in glass bowls (4L) in F2 medium (Sigma-Aldrich), under controlled conditions (18°C, 12/12 h photoperiod in a thermostatic chamber), and the biomass produced after 10 days was filtered on glass fiber filters (GFF) and freeze-dried. To produce benthic diatoms (*Cocconeis* spp., *Navicula* sp., and *Diploneis* sp.), monoclonal cultures were cultivated in sterilized Petri dishes (14 cm diameter), each containing 100 mL of F2 medium. After 15 days of growth in a thermostatic chamber (18°C, 12/12 h photoperiod) Petri dishes were opened, the culture medium was drained, and the dishes were washed twice with distilled water, then freeze-dried and scraped with a metal blade, to collect the biomass produced. Part of the dried materials produced by both planktonic and benthic diatoms was included in composed foods, according to the methods reported below. Another part was used for extraction of active compounds.

For this purpose, 60mg of freeze-dried diatoms were added to 2mL of methanol (MeOH) and homogenized (20°C) using a 2.5 mL potter. The extraction was repeated 3 times and the crude extracts obtained were pooled prior to partitioning between hexane and 10% aqueous MeOH solution. The MeOH phase was then diluted to 40% aq. MeOH (Jüttnet et al. 2000) and extracted with CH₂Cl₂. Evaporation of the solvents (Büchi rotavapor) and lyophilization of the aqueous solution permitted us to obtain hygroscopic solids that were stored in a cold (−20°C) and dry environment. The solid residue (mainly empty frustules of diatoms) obtained after the extraction was centrifuged (10 min at 3000 rpm), dried, and included in foods, to check for the presence of any residual activity.
Finally, aldehydes previously identified (Romano et al. 2003; Ianora et al. 2004) in planktonic diatoms (2-trans-4-trans-decadienal; 2-trans-4-trans-octadienal) were obtained (Sigma-Aldrich) and incorporated into the shrimp food. Bioassays of diatoms, of their fractions, and of the aldehydes obtained as reported above, indicated where apoptotic activity was principally located.

The materials to be tested (whole diatoms, their extracts, and the diatom aldehydes) were mixed into a basic composite food, containing 40% dried algae (Enteromorpha sp.), 40% of dried Artemia salina enriched with fatty acids (PUFA, commercially sold as “SHG Enriched Artemia”), and 20% of enriched Spirulina flakes (manufactured by SHG inc., www.superhigroup.com). Small pellets (5 mg each) containing known amounts of the diatoms and their extracts were prepared according to the experimental plan reported in Table I. Each of the organic fractions extracted from 60 mg of dried diatoms (i.e., MeOH fraction, CH$_2$Cl$_2$ fraction, hexane fraction, and solid residue) was mixed with 300 mg of dry food, then partitioned into 5 mg pellets. Additionally, 60 mg of dried diatoms for each species of benthic and planktonic microalgae were included into 300 mg dry food and partitioned in 5 mg pellets. Finally, 3 μL of a 2 mg mL$^{-1}$ methanol solution of the two reference aldehydes were added to 300 mg dry food and partitioned into 5 mg pellets.

Mature H. inermis females were sampled in the Posidonia oceanica meadow off Lacco Ameno d’Ischia (Gulf of Naples, Italy), transported to the laboratory, and cultured until

| Table I. Experimental plan consisting of a control and 27 treatments, each with 3 replicates and 25 individuals. |
|---------------------------------|---------------------------------|
| Control                         | none                            |
| Cocconeis neothumensis           | 60 mg dry diatoms               |
| Cocconeis scutellum parva       | 60 mg dry diatoms               |
| Diploneis sp.                    | 60 mg dry diatoms               |
| Navicula sp.                     | 60 mg dry diatoms               |
| Skeletonema costatum             | 60 mg dry diatoms               |
| 2-trans-4-trans-decadienal      | 6 μg of the S. costatum aldehyde|
| 2-trans-4-trans-octadienal      | 6 μg of the S. costatum aldehyde|
| C. neothumensis MeOH fraction    | Methanolic fraction from 60 mg diatoms |
| C. neothumensis hexane fraction  | Hexane fraction from 60 mg diatoms |
| C. neothumensis CH$_2$Cl$_2$ fraction | CH$_2$Cl$_2$ fraction from 60 mg diatoms |
| C. neothumensis residue          | Solid residue collected after the extraction of 60 mg diatoms |
| C. scutellum parva MeOH fraction | MeOH fraction from 60 mg diatoms |
| C. scutellum parva hexane fraction | Hexane fraction from 60 mg diatoms |
| C. scutellum parva CH$_2$Cl$_2$ fraction | CH$_2$Cl$_2$ fraction from 60 mg diatoms |
| Cocconeis scutellum parva residue | Solid residue collected after the extraction of 60 mg diatoms |
| Diploneis sp. MeOH fraction      | MeOH fraction from 60 mg diatoms |
| Diploneis sp. hexane fraction    | Hexane fraction from 60 mg diatoms |
| Diploneis sp. CH$_2$Cl$_2$ fraction | CH$_2$Cl$_2$ fraction from 60 mg diatoms |
| Diploneis sp. residue            | Solid residue collected after the extraction of 60 mg diatoms |
| Navicula sp. MeOH fraction       | MeOH fraction from 60 mg diatoms |
| Navicula sp. hexane fraction     | Hexane fraction from 60 mg diatoms |
| Navicula sp. CH$_2$Cl$_2$ fraction | CH$_2$Cl$_2$ fraction from 60 mg diatoms |
| Navicula sp. residue             | Solid residue collected after the extraction of 60 mg diatoms |
| S. costatum MeOH fraction        | MeOH fraction from 60 mg diatoms |
| S. costatum hexane fraction      | Hexane fraction from 60 mg diatoms |
| S. costatum CH$_2$Cl$_2$ fraction | CH$_2$Cl$_2$ fraction from 60 mg diatoms |
| S. costatum residue              | Solid residue collected after the extraction of 60 mg diatoms |
larvae were released (Le Roux 1963). An experimental larval culture process was devised according to Zupo and Messina (2007) to obtain a sufficient number of postlarvae. Postlarvae obtained from all females were pooled and divided in groups of 25 individuals in Petri dishes to obtain 27 treatments (Table I), each consisting of three replicates. The experimental control was the basic composite food. At the end of the experiment (25 days) all mature postlarvae were sacrificed, fixed, and examined under the dissecting microscope, to record the total length and to collect the second pleopod, in order to determine sex ratios. The ratio ‘number of females/total number of mature individuals’ ($F_{tot}$) was calculated for each treatment, as well as the mortality rates and the mean size reached by adult shrimp at the end of the experiment. Results were statistically analyzed to check if the planktonic diatoms may have an effect comparable to that of benthic diatoms and also to identify the fractions exhibiting the highest activity. Two-way ANOVA with Bonferroni post-test was performed using Prism software (version 4.00 for Mac, GraphPad Software, San Diego California USA) to evaluate significant differences among treatments. A One-way ANOVA with multiple comparison test was used to check the differences between the control and each treatment. $F_{tot}$ ratios between control and treatments were compared by means of a z-test on proportions.

**Results**

The larval growth lasted on average 30 days, while the postlarval growth lasted 25 days. At this age most individuals were mature and they were sacrificed. Low mortality rates for postlarvae were observed for most treatments with freeze-dried diatoms, with the exception of $S. \text{costatum}$ (10.0%) and $C. \text{scutellum parva}$ (11.7%; Figure 1). All the remaining treatments with whole diatoms, as well as the control, exhibited mortality rates lower than 10%. The highest $F_{tot}$ ratio (number of females on the total number of individuals), indicating high efficiency in the production of primary females, was exhibited by $C. \text{neothumensis}$ (61.1%; Figure 1). A high $F_{tot}$ ratio, significantly different with respect to the control, was also exhibited by the treatment with $C. \text{scutellum parva}$. This is in accordance with the results of Zupo and Messina (2007). In contrast, the $F_{tot}$ ratios obtained with the treatments $Diploneis$ sp. (46.1%), $Navicula$ sp. (39.5%) and $S. \text{costatum}$ (31.3%) were not significantly different in respect to the control (31%; Figure 1).

The size reached at the end of the experiment (Figure 2) adds some insights to the previous information. Two-way ANOVA indicated significant differences among both diatoms and extracts ($p < 0.0001$), with significant statistical interactions between factors. The greatest sizes were reached by $H. \text{inermis}$ under the treatments with $Diploneis$ sp. (7.0 mm) and $Navicula$ sp. (6.9 mm). The average size observed in control individuals was 6.4 mm, which is not significantly different from the size obtained with the benthic diatoms $C. \text{scutellum parva}$ and $C. \text{neothumensis}$. A significant difference, with respect to the controls, was observed only for the treatment with $S. \text{costatum}$ (5.9 mm). Very complex patterns were shown by the size reached under various treatments with solvents (Table II), but the greatest size was reached in individuals under the treatment with fresh and the solid residue of $Diploneis$ sp. (7.4 and 7.2 mm, respectively) while the smallest size was reached in individuals under the treatment with the freeze-dried $S. \text{costatum}$. The mortalities observed in the treatments with aldehydes were low as well (less than 10.0%) but in this case no significant differences were observed between the $F_{tot}$ ratios in the two treatments and in
Figure 1. Mean mortality (% of dead individuals at the end of the 25-day experiment) and percent of females on the total number of individuals (F/tot) of *Hippolyte inermis* in three replicates, ordered according to the efficacy of treatments. Only treatments with freeze-dried diatoms are considered here. An asterisk denotes the treatments significantly different from the controls.

Figure 2. Mean size (mm) + 1 SD ($n = 3$) reached by *Hippolyte inermis* postlarvae at the end of the 25-day experiment. A black bar denotes the control.
Table II. Mean size (mm) reached in each treatment and for each solvent, followed by standard deviations (SD) and number of individuals (N). N (75 at the start of experiment) varies according to the mortality observed in each treatment. All fractions were compared to the same control. CTRL = control; Fresh = whole diatoms; F-D = freeze-dried diatoms; Met = methanol fraction; Hex = Hexane fraction; CH\textsubscript{2}Cl\textsubscript{2} = CH\textsubscript{2}Cl\textsubscript{2} fraction; Res = solid residue.

|                | Fresh | SD  | N  | F-D | SD  | N  | Met | SD  | N  | Hex | SD  | N  | CH\textsubscript{2}Cl\textsubscript{2} | SD  | N  | Res | SD  | N  |
|----------------|-------|-----|----|-----|-----|----|-----|-----|----|-----|-----|----|                              | -----|----|-----|-----|----|
| Control        | 6.43  | 0.02| 57 | 6.43| 0.02| 57 | 6.43| 0.02| 57 | 6.43| 0.02| 57 |                              | 6.43 | 0.02| 57 |
| C. neothumensis | 6.40  | 0.25| 49 | 6.73| 0.32| 54 | 6.57| 0.34| 60 | 6.60| 0.16| 49 | 6.59                          | 0.16 | 52 | 6.54| 0.23| 58 |
| C. scutellum parva | 7.06  | 0.16| 51 | 6.59| 0.23| 53 | 6.27| 0.02| 58 | 6.82| 0.45| 52 | 6.77                          | 0.24 | 56 | 6.73| 0.15| 50 |
| Diploneis sp.  | 7.45  | 0.67| 37 | 7.01| 0.02| 57 | 6.74| 0.57| 43 | 6.53| 0.17| 36 | 7.10                          | 0.06 | 37 | 7.25| 0.77| 36 |
| Navicula sp.   | 6.79  | 0.09| 38 | 6.88| 0.28| 38 | 6.64| 0.12| 37 | 6.67| 0.02| 35 | 6.53                          | 0.04 | 34 | 6.96| 0.26| 37 |
| Skeletonema costatum | 6.21  | 0.20| 32 | 5.98| 0.08| 36 | 6.50| 0.12| 32 | 6.50| 0.25| 40 | 6.19                          | 0.31 | 29 | 6.68| 0.26| 32 |
the control (Figure 3). The highest percentage of females was obtained with the ‘decadienal’ treatment (47.5%).

The results obtained with the diatom extracts showed a complex pattern but, on average, the methanolic fractions appeared to be the most active in most treatments (Figure 4). The solid residues were also active however (large F/tot ratios), especially in the treatments with some diatoms (S. costatum, C. scutellum parva, and C. neothumensis). This indicates that the solvents used for the extraction were not efficient. However, both the hexane and dichloromethane fractions exhibited a low efficacy in promoting the development of primary females, and their activities were inversely proportioned to the one of solid residue (Figure 4). It is worth noting that the activity of the methanolic fractions was higher in the treatments with C. scutellum parva, Navicula sp. and C. neothumensis, thus demonstrating the highest efficacies (Figure 4) as a whole. A high F/tot ratio was exhibited also by the MeOH fraction of Diploneis sp., which was characterized by the lowest activity of the solid residue. In contrast, the efficacy of the methanolic fractions was low in S. costatum.

Discussion

The results obtained with the dry diatom bioassays confirm the activity of C. neothumensis and C. scutellum parva in promoting the development of primary females (Zupo and Messina 2007). They also demonstrate that the planktonic diatom S. costatum exhibits a negligible effect on the sex reversal of the shrimp H. inermis. Since previous investigations (Zupo 2000; Zupo and Messina 2007) indicated that the development of primary females is due to a very early disruption by apoptosis of the androgenic gland (AG) of young postlarvae, followed by the death of the testis and the development of an ovary.
(Charniaux-Cotton 1954; Austin and Meewan 1999; Khalaila et al. 2002), we should infer that *C. neothumensis* and *C. scutellum parva* contain compounds able to specifically trigger the apoptosis of the AG in *H. inermis* postlarvae, while the planktonic diatom *S. costatum*, previously demonstrated to induce apoptosis in embryos and larvae of planktonic copepods (Poulet et al. 1994; Ianora et al. 1995), does not contain the same compound.

In fact it is evident that the disruption of the testis and the formation of an ovary should be preceded by the disappearance of the AG (Payen 1983; Nagamine et al. 1980; Khalaila et al. 1999), since in *H. inermis* intersex individuals were never observed (Sagi et al. 1997, 2002; Cobos et al. 2005). The compounds occurring in *S. costatum* are apparently characterized by a specific toxicity on *H. inermis*, as demonstrated by the low size reached at the end of the experiment with this planktonic diatom. However, the toxic effect was not due to the apoptotic compounds (aldehydes) previously found in this diatom. The mortality recorded in the treatments with octadienal aldehyde was lower than the mortality recorded in the treatment with the whole diatom.

The data on the size reached at the end of the experiment with fractions may add some information to this puzzling question. Shrimps exposed to the CH$_2$Cl$_2$ fraction of *S. costatum* exhibited the smallest size and the highest mortality. This indicates that the toxic compound present in the planktonic diatom is selectively extracted by this solvent (Jüttner 2001). Recent studies (d’Ippolito et al. 2004) indicated that other compounds, besides aldehydes, may be responsible for the effects observed in the planktonic copepods and that these compounds could be selectively extracted by CH$_2$Cl$_2$. 

![Figure 4. Mean percent of females on the total individuals of *Hippolyte inermis* (F/tot) ordered according to the efficacy of treatments. Treatments with extracts are considered here. A black bar denotes the control. An asterisk denotes the treatments significantly different from the control. Met = methanol extract; Res = solid residue after the extraction; Hex = Hexane extract; CH2Cl2 = CH$_2$Cl$_2$ extract.](image-url)
In contrast, the largest size was reached in the treatment with *Diploneis* sp. and in the treatment with its solid residue. This seems to indicate that the solid residue of diatoms still contains some compounds or feeding principle useful to promote growth of shrimp. The final size reached by shrimps under the treatments with the two species of benthic *Cocconeis* is not, however, significantly different from the size of shrimps under the control diet or fed with other benthic diatoms. This indicates that the process of apoptosis disrupting the AG of young shrimps is not due to a toxic effect, but rather to a specific activity influencing their physiology (Zupo 1994). No differences in food acceptance were observed among treatments and all cultured shrimps produced similar quantities of fecal pellets. These observations mean that size differences cannot be ascribed to the amount of ingested food. In addition, our data demonstrated that the main apoptotic compounds found in planktonic diatoms, i.e., octadienal and decadienal aldehydes, have negligible effects on the sex maturation of *H. inermis* and, therefore, these compounds should be different from those responsible for the effects on the sex ratio produced by benthic diatoms (Zupo et al. 2000).

The results obtained by administering the fractions obtained from various benthic diatoms should be considered indicative, but not conclusive, in starting the characterization of the compounds responsible for the effect on the sex ratio of *H. inermis*. A high residual activity was found in the solid fraction of diatoms after the extraction with different solvents, and this indicates the need for further investigations with different sets of solvents. However, the methanolic fraction was significantly active (in respect to the control) in the case of the effective diatoms *C. neothumensis* and *C. scutellum parva*. The activity of both hexane and dichloromethane fractions, in all diatoms, was low (*F/tot* < 46 and 43%, respectively) and their effects were not significantly different from the control group. It is also evident that the activity of the methanolic fraction of *S. costatum*, whose effect on sex maturation was negated by other experimental manipulations (freeze dried biomasses), was low and comparable to that of the hexane and dichloromethane fractions. This confirms the absence of activity of this planktonic alga and represents a further indication of the ability of methanol to segregate at least part of the active compound (since another important part, apparently, remains in the solid residue). In contrast, *Diploneis* sp. exhibiting slight activity as a whole diatom (Figure 1) demonstrated the highest methanolic activity and the lowest activity of the solid residue (‘Res’ in Figure 4). This may indicate that the residual activity in the solid fraction is linked to some physical properties of diatoms and that the activities of the solid residue and of the methanolic fraction are inversely proportioned, since an effective extraction may move most of the activity from the solid part to the ‘MeOH’ fraction (Fink et al. 2006).

Taking into account that the effects of *S. costatum* on the sex of our model shrimp were not significant within the planned factorial experiment and that benthic diatoms triggered a significant shift to female sex, we conclude that the apoptotic compounds present in the diatoms examined are different. Moreover, *S. costatum* promoted a significant increase of mortality and a lower growth of postlarvae, thus demonstrating that, in the benthos as well as in the plankton, this diatom is characterized by a high toxicity (d’Ippolito et al. 2004). The results obtained with freeze-dried diatoms were confirmed by the tests with aldehydes, demonstrating high mortality, low growth rates, and non-significant effects on the sex ratio of our test shrimps. The solvent apparently extracting the active compound was methanol, although some activity remained in the solid residue. This observation indicates that, besides a difficulty of extraction, the active factor should be at least a partially polar compound (Blom and Jüttnet 2005). Further research, using different sets of solvents, will be necessary to fractionate and further characterize the active compound occurring in some
benthic diatoms, absent in planktonic diatoms. We suggest that the compound is probably useful for biotechnological applications (Schwartsmann et al. 2001, 2003), due to its specific activity (Bongiorni and Pietra 1996) on the androgenic gland of *H. inermis*. The apoptotic activities promoted by planktonic and benthic diatoms, both producing effects on the physiology of various crustaceans (reducing the size of recruitment in planktonic copepods, stabilizing the natural populations in benthic crustaceans) are analogous, because they are based on different chemical compounds.

Acknowledgments

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References


How do dietary diatoms cause the sex reversal of the shrimp
Hippolyte inermis Leach (Crustacea, Decapoda)

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Abstract Hippolyte inermis Leach 1915 is a protandric shrimp largely distributed in Posidonia oceanica meadows and other Mediterranean seagrasses. Previous studies demonstrated several physiological peculiarities, such as absence of female gonadic buds in adult males (the new female gonad is produced starting from few undifferentiated cells), the consequent absence of an ovotestis, 2 yearly periods of reproduction with different population structures (a spring outburst producing both males and primary females, and a fall reproduction producing mainly males), and a process of sex reversal influenced by the diatom food ingested. We performed several laboratory analyses to compare the effects of various species of benthic diatoms, in order to test the effect of different diatoms and provide information on the mechanism of action of the ingested compounds. In addition, we performed molecular tests (TUNEL) and TEM observations, to check the hypothesis that the effect of benthic diatoms may be mediated by a process of apoptosis acting on the male gonad. The results obtained allowed for a ranking of a series of benthic diatoms according to their effects on sex reversal, and a confirmation of the striking effect of Cocconeis sp. diatoms, which are able to trigger the appearance of primary females. We also demonstrated the presence of apoptosis both in the male gonad and in the androgenic glands of postlarvae. The effect is species specific, strictly localized to the male gonad and androgenic gland, and limited to a very short period of time, from the 5th to the 12th day of postlarval development.

Introduction

The shrimp Hippolyte inermis Leach, 1815 lives in shallow waters of the Mediterranean Sea and along the Atlantic coast of Spain (Zariquiei Alvarez 1968). It forms stable populations in seagrass meadows (Gambi et al. 1992), mainly in Posidonia oceanica and Cymodocea nodosa (Guillen Nieto 1990). Most individuals exhibit a green mimic colour (Bedini et al. 1997). Investigations by Reverberi (1950) and Veillet et al. (1963) demonstrated individuals experiencing a male stage prior to switching to female (i.e. protandric sex reversal; Gherardi and Calloni 1993). We also know (Le Roux 1963; Regnault 1969) that juvenile diet shifts from zooplankton (larvae) to microalgae and microzoobenthos (settled postlarvae). Sex differentiation occurs at a size of 5–7 mm (Veillet et al. 1963); sex reversal was observed in individuals of 10–13 mm, corresponding to an age of 7–12 months (Zupo 1994). Not all individuals exhibit sex reversal. In fact, young females of 5–6 mm length are present in natural populations. They are smaller than any male and are produced by direct differentiation. Large females, originating from sexual inversion, were designated as alpha females, while small females, directly developed, were designated as beta females (Zupo 1994). Two main periods of recruitment, spring and fall, were detected in the life cycle of H. inermis. Individuals born in spring grow quickly and develop as either females or
males, while individuals born in fall grow slowly and develop as males, changing sex in the next spring. The spring period of maximum abundance of beta females in natural populations corresponds to a massive ephytic production in the leaf stratum of *P. oceanica* (Mazzella and Buia 1989; Zupo 1994).

*Hippolyte inermis* is characterized by a singular feature (Reverberi 1950): female gonads are not produced starting from buds (as in other sex-reverting invertebrates; Charniaux-Cotton 1960) and an ovotestis is never observed (as it occurs in other decapods). They are built up from undifferentiated cells, and the male gonad cannot be influenced by hormones produced by an ovary during its development (Reverberi 1950; Katakura 1989). The absence of an ovotestis was recently observed also by Cobos et al. (2005). Based on this observation these authors concluded that the species should be gonocoric. Actually, the absence of an ovotestis (yet observed by Reverberi in 1950) is not sufficient to negate sex reversal, well demonstrated by previous investigations by Veillet et al. (1963) and Zupo (1994, 2000), since it is known (Reverberi 1950) that this shrimp is protandric, not hermaphroditic (i.e. shift of sex from male to female proceeds in the absence of an ovotestis). Moreover, our present historical investigations demonstrate that the disruption of testis and androgenic gland, quickly followed by the production of an ovary, is a very rapid process, lasting about 1 week and concluded within a single ecdisyal cycle.

According to the previous observations, the effect of compounds contained in dietary diatoms (*Cocconeis* sp., according to Zupo 2000) should be directed towards the male gonad, destroying it during its development (Zupo 1994). We hypothesized (Zupo 2000) that the process of sex reversal may proceed through apoptosis (programmed cell death; Raff 1998) of the male gonad. In fact, due to the absence of gonadic buds producing hormonal substances and depressing the male gonad physiology (as it occurs in other protandric crustaceans; Charniaux-Cotton and Payen 1988) the ingested diatoms seemingly contain compounds that selectively destroy cell populations, inducing their suicide. The action of the apoptotic compounds should be species-specific and extremely selective for the male gonad. It should trigger the quick death of cells naturally programmed to die about 12 months after hatching. The target tissues should be those of the male gonad and, probably, the androgenic gland (AG).

In fact, the regulation of the male reproductive system of decapod crustaceans is controlled by the androgenic gland (AG; Sagi et al. 1997b; Sagi and Khalaila 2001). The initiation, completion and intensity of spermatogenic activity are regulated by circulating AG hormone (Charniaux-Cotton and Payen 1988). The AG hormone was purified in Isopoda (Ohira et al. 2003) but has not yet been isolated and characterized in decapod crustaceans. Spermatogenesis starts only when the AGs are fully developed in certain decapod species (Payen 1973; Taketomi et al. 1996). On the other hand, in the male prawn *Macrobrachium rosenbergii* (Nagamine et al. 1980) and in intersex individuals of the Australian red claw crayfish *Cherax quadricarinatus*, removal of the AG leads to cessation or regression of spermatogenesis (Khalaila et al. 1999) and to development of female primary and secondary sex characters (Sagi et al. 1997a, 2002). The expression of the vitellogenin gene was detected by RT-PCR in the hepatopancreas of the AG ablated intersex of various crustaceans (Sagi et al. 2002). There is an ongoing effort to find chemical agents responsible for the regulation of the above shifts from maleness to femaleness in order to control sexual plastic processes in commercially important crustaceans and to determine the sex of offspring groups.

The influence of diatoms on the reproductive ecology and life cycle of other crustaceans (Miralto et al. 1995; Ianora et al. 1995), mainly copepods (Poulet et al. 1994) has been demonstrated. The production of diatom compounds, detrimental to the development and survival of grazers, has major impacts on secondary production (Miralto et al. 1996, 1999). Other effects are hypothesized, however, in *H. inermis* (Adiyodi and Adiyodi 1970; Zupo 1994), according to co-evolutionary processes: it is largely adapted to the life in *P. oceanica* (d’Udekm d’Acoz 1996) and the toxic effect of diatoms is translated into a spring signal for the development of beta females, whose presence is a crucial factor for maintaining a constant sex ratio (Zupo 1994; Buia et al. 2000).

Resuming the previous points, it has been shown that: (a) the ingestion of *Cocconeis* sp. diatoms induces the development of primary (beta) females (Zupo 1994); (b) the same mechanism of action occurs both in the laboratory and in the field (Zupo 2001); (c) the process takes place in postlarvae, presumably in the first phases of sex maturation (Zupo 2000); (d) it could hardly be mediated by female hormones (Reverberi 1950; Katakura 1989; Khalaila et al. 2002). Therefore, the objective of this study was to determine the mechanism by which benthic diatoms influence sex determination in *Hippolyte inermis*. We cultured various species of benthic diatoms and included them in the diets of *H. inermis* postlarvae. We studied how organs involved in sex determination are affected by different diets using an in situ cell death detection kit based on TdT-mediated dUTP Nick End Labelling technique.
(TUNEL) provided by Roche Diagnostics GmbH. In addition, the presence of apoptosis in specific organs of the treated shrimps was checked by Transmission Electron Microscopy (TEM) and light microscopy observations on testes and AG thin sections.

**Material and methods**

**Diatom collection and culture**

Diatom collections were performed by displacing metal panels, covered with a silicon polymer, close to a *Posidonia* meadow, at 1.5 m depth. The low surface tension of silicon coatings facilitates the selection of diatoms of the genus *Cocconeis*, which are characterized by higher adhesive power. The special coating allowed for sampling sufficient amounts of intact microalgae for the next phases. The surface of the panels was sampled weekly in areas of 2 cm², by means of a cover slide gently scraped on the wet surface. Each sample was quickly transported to the laboratory, dispersed in 5 ml clean seawater and analysed under an inverse optical microscope. Diatoms of the genus *Cocconeis* were collected by micropipettes, with the aid of a Leica micromanipulator, and individually transferred in sterilized Petri dishes, containing Guil-lard’s “f2” with silicates (Sigma-Aldrich Biochemicals) as a culture medium. After several transfers and selections were conducted, using a micromanipulator at 5-day intervals, monoclonal cultures of diatoms were obtained. Samples of these cultures were collected, filtered, fixed on microscope stubs and gold-sputtered for Scanning Electron Microscope (SEM) examination and species identification (De Stefano et al. 2000). These techniques allowed for isolating live strains of the main species of *Cocconeis* living in *Posidonia* meadows, i.e. *C. scutellum scutellum*, *C. scutellum parva*, *C. neothumensis*, *C. dirupta*. In addition, monoclonal cultures of *Navicula* sp., *Amphora* sp. and *Diploneis* sp. were obtained and used as a control of the bioactivity, by means of bioassays on living shrimps. The obtained monoclonal cultures were maintained in thermostatic chambers at 18°C, under constant irradiance (140 µE), with a 12/12 h photoperiod, and transferred at 15-day intervals.

**Rearing of larvae and bioassays on *H. inermis* postlarvae**

Ovigerous females of *H. inermis* were collected in the field (*Posidonia oceanica* meadow in Lacco Ameno d’Ischia) using the technique described by Zupo (2000) and reared in the laboratory, individually, in aerated 2 l bowls, until larvae were released. Larvae were collected on 60 µm nets and subjected to the standard procedure (Zupo 2000) for production of postlarvae, in approximately 20 days. The technique consists of the culture of larvae in 11 vessels (containing 800 ml of filtered and UV sterilized seawater) at a density of 1 larva per 10 ml of culture solution, containing three nauplii of *Artemia salina* (450 µm length) and two *Brachionus plicatilis* per ml. The culture vessels, aerated by the use of an air pump, were maintained in a thermostatic chamber at a constant temperature of 18°C, with a 12/12 photoperiod. The culture media was renewed at 2-day intervals, after filtering of larvae on a 60 µm net and counting survivors.

Postlarvae were transferred to 12 cm Petri dishes (filled with 400 ml of filtered seawater) in stocks of 25 individuals. Several (from 2 to 9) replicate Petri dishes (25 ind. each) were used for each treatment, according to the availability of fresh diatoms for each diet (see Table 1). The postlarvae of each treatment were fed on fresh diatoms grown on 12 cm Petri dishes. Postlarvae produced in different vessels and from different females were pooled prior to the start of the bioassays, to randomize any difference due to maternal influences. Prior to starting the bioassay experiments on shrimps, diatoms of each selected species were transferred into individual Petri dishes of 12 cm containing 70 ml of culture media (Guillard’s f2). An almost continuous layer of diatoms covered the bottom of the dishes after 15 days. At this time, the culture media was drained, and each Petri was filled with 400 ml of filtered seawater, in order to host postlarvae, which were allowed to feed on the algae adherent to the bottom. A composed dry food (Tetra AZ 300 artificial plankton for shrimps) was used as a diet integrator, since it is known that these shrimps need both animal and plant feeding items (Zupo 2001). Moreover, green macroalgae (*Enteromorpha* sp., cultured in the laboratory) were tested as an additional control. Eight

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<th>Table 1</th>
<th>List of the diets considered for the bioassays and respective number of replicates performed</th>
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<td>Diet</td>
<td>Content</td>
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<td>F</td>
<td>Dry food</td>
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<tr>
<td>M</td>
<td>Dry food and <em>Enteromorpha</em> sp.</td>
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<tr>
<td>CSP</td>
<td><em>Cocconeis scutellum parva</em> and dry food</td>
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<td>CSS</td>
<td><em>Cocconeis scutellum scutellum</em> and dry food</td>
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<td>CN</td>
<td><em>Cocconeis neothumensis</em> and dry food</td>
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<tr>
<td>N</td>
<td><em>Navicula</em> sp. and dry food</td>
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<td>A</td>
<td><em>Amphora</em> sp. and dry food</td>
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<td>D</td>
<td><em>Diploneis</em> sp. and dry food</td>
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<th>Nr of replicates</th>
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different species of diatoms, compared to the effect of negative controls (macroalgae and dry food) as reported in Table 1.

One individual was sampled from each replicate of each treatment and frozen (−80°C) at 4-day intervals (alternated among the replicates of each treatment, to obtain 2-day lagged data). These individuals were then subjected to TUNEL and TEM analyses. The growth rates of larvae in each treatment were followed by measuring a subset of 20 individuals and by observing the sex maturation (shape of the first two pleopods) in narcotized postlarvae (30 s immersion in a 80 ppm solution of chloroform in seawater, obtained by solving 16 μl chloroform in 200 ml of filtered seawater).

Analysis of sex

Mature postlarvae (in this species, the appearance of secondary sexual characters corresponds to sexual maturity: Zupo 2000) were frozen after 25 days and examined under a dissecting microscope, to record their total length and to collect the second pleopod. The examination of the pleopod II under an optical microscope allowed us for the determination of their sex (presence or absence of the appendix masculina). When complete data sets were obtained, statistical analyses (ANOVA on biometric data and Z-test on proportions of males and females) were performed, to evaluate the significance of differences between tests and controls, assess the efficacy of each diatom species and rank their biological activity.

Detection of apoptosis

At the end of the experiment, individuals frozen at various developmental stages (previously collected at 4-day intervals, as above mentioned) were subjected to the TUNEL analysis (Roche Molecular Biochemicals Kit), for the rapid detection of apoptosis, according to the standard procedure (Romano et al. 2003). This molecular technique identifies DNA strand breaks by labelling 3′-OH termini with modified (fluorescein labelled) nucleotides in an enzymatic reaction. Therefore, the technique was here applied to establish whether the abundance of females in the treatments subjected to diatom foods was due to an early apoptosis (programmed cell death) of the testis tissues.

Each individual to be tested was fixed in 4% paraformaldehyde and its abdomen was ablated, to permit a better penetration of the reagents in the carapace (where the gonads and the AGs are located). After 1 day, each individual was rinsed in phosphate buffered saline (PBS) 1x solution, to wash out the fixative, then transferred in citrate buffer (pH 6) and in chitinase, to digest the carapace chitin; finally it was rinsed again in PBS and immersed in Triton-X 100 overnight, to permeabilize its tissues and allow for a better penetration of dyes (see Romano et al. 2003 for a detailed description of the technique).

The third day, all individuals were rinsed twice in PBS and incubated in the TUNEL reagents, to label the DNA fragments. One positive control was previously treated with DNase (to check the effectiveness of TUNEL treatment), while a negative control was immersed in the TUNEL label solution, without immersion in the terminal deoxynucleotidyl transferase enzyme (TdT), catalysing the polymerization of nucleotides at the 3′-OH end of the DNA fragments (to check for the absence of fluorescence). After 90 min of incubation at 37°C and three rinses in PBS, to wash out the fluorescent reagent, all samples were ready for observations. The treated individuals were examined under a UV complanar microscope (Leica Z16 APO) or under a laser confocal microscope (Zeiss 410 He/Ne laser 543 nm), to obtain 3-D images and record any fluorescence due to apoptosis. The results of molecular analyses were confirmed by observations at the TEM of thin slices of the same individuals, after post-fixation with 1% osmium tetroxide and embedding in Epon 812 resin.

In addition, histological sections (5 μm) of several male and female shrimps were obtained (after fixing in Carnoy and embedding in paraflin) and stained according to the ematoxilin-eosin technique, to map the shrimp morphology and check the position and shape of the fluorescent structures detected by TUNEL analyses.

Results

In total, 37 adult females of Hippolyte inermis were sampled and each produced a mean of 75 larvae. Pooling the productions of all females, 2,784 larvae were obtained and subjected to growing experiments. During larval growth (lasting meanly 24 days; Fig. 1) 20.54% mortality was recorded, while during the postlarval growth (lasting meanly 25 days), 15.11% (±10.28) mortality was observed. The mean size at the reaching of postlarval stage was 3.4 ± 0.4 mm (Fig. 1). All individuals were subjected, during the larval phase, to the same treatment and cultured contemporaneously in the same thermostatic chamber. In contrast, different growth rates were recorded among treatments
performed on postlarvae (Fig. 1; $R^2 > 0.9$), with Cocconeis scutellum scutellum and Navicula sp. exhibiting the highest slopes of the growth curves. Most treatments exhibited a sex maturation period of 25 days from the production of postlarvae (24 days after hatching) to the appearance of secondary sexual characters (49 days after hatching), but the treatments with Amphora sp. and dry food lasted 30 days.

The postlarvae sacrificed at the end of each treatment had a total mean length of 6.72 mm ($\pm 0.37$), but differences were observed among diets (Fig. 2). Amphora sp. exhibited the smallest size (5.88 mm), while Navicula sp, Cocconeis scutellum scutellum and Cocconeis neothumensis exhibited the largest sizes (7.01, 7.00 and 6.97 mm, respectively). Differences among slopes of growth curves were significant (ANOVA, $P < 0.01$) only for Amphora sp. and dry food. The average percentage of sexually undetermined (and sexually immature) postlarvae at the end of the experiments was 37.6% ($\pm 25.4$), but differences were observed among replicates (Fig. 2). Moreover, the treatments “Dry food”, Cocconeis neothumensis and Cocconeis scutellum scutellum exhibited the lowest percentage of sexually undetermined postlarvae (5.75, 10.00 and 24.97%, respectively), while the treatments Amphora sp., Diploneis sp. and Cocconeis scutellum parva displayed the highest percentages of sexually undetermined postlarvae (63.74, 45.56 and 37.69%, respectively). Also the mortality during postlarval growth was low, on average, but differences were observed among treatments (Fig. 2), with Cocconeis neothumensis (35.75%), Amphora sp. (27.60%) and macroalgae (14.88%), respectively, producing the highest mortalities.

The female/matures ratio, indicating the efficacy of each species of diatoms for triggering the development of beta females, exhibited large variations among the replicates as well (Fig. 3). Significant differences, however, were observed between several treatments. Considering dry food (F) and macroalgae (M) as the negative controls, Navicula sp. (N) and Amphora sp. (A) shared similar percentages of females on the total of mature individuals, which indicate absence of activity on the sex reversal process. In contrast, other species of diatoms exhibited a significant effect, triggering the production of larger percentages of beta females (Fig. 3). In particular, Diploneis sp., Cocconeis neothumensis and C. scutellum parva exhibited the highest

![Fig. 1](image1.png)

**Fig. 1** Larval (first 25 days after hatching, left plot) and postlarval (next 20 days, right plots) growth rates expressed as size of larvae (left axis) and postlarvae (right axis) in mm. A single curve expresses larval growth, because all larvae were cultured in identical conditions. Several dotted lines express postlarval growth, according to individual treatments. The names of treatments refer to the acronyms reported in Table 1.

![Fig. 2](image2.png)

**Fig. 2** Percentage of immatures (sexually undetermined, as explained in the text), mortality and size (mm) reached by shrimps at the end of the experiment, according to each treatment (the names on the x-axis refer to the acronyms reported in Table 1). The standard deviations among replicates of the same treatment are reported. Asterisks on the bars indicate the significance of differences against the controls (*$P < 0.05$; **$P < 0.01$) determined by means of Z-test. The sizes of treatment groups ($N$) are reported in Fig. 2.

![Fig. 3](image3.png)

**Fig. 3** Females/mature ratios obtained for each diet, ranked according to effectiveness. The names of the treatments are reported on the x-axis according to the acronyms contained in Table 1. The standard deviations among replicates of the same treatment are reported. Asterisks on the bars indicate the significance of differences against the controls (*$P < 0.05$; **$P < 0.01$) determined by means of Z-test. The sizes of treatment groups ($N$) are reported in Fig. 2.
female/mature ratios (0.62, 0.55 and 0.53, respectively). Z-tests performed to compare the female/mature ratios obtained in controls and tests demonstrated significant differences (rejection of the null hypothesis, absence of differences, at \( P < 0.05 \)) for *C. neothumensis* and highly significant differences (\( P < 0.01 \)) for *C. scutellum parva*, and *Diploneis* sp.

The TUNEL technique, applied to test and control shrimps sampled during the culture of postlarvae, indicated presence of apoptosis in various organs of shrimps feeding on *C. neothumensis*, *C. scutellum parva* and *Diploneis* sp. (Fig. 4). In particular, evidence of apoptosis was detected in the AG glands (Fig. 4b); moreover, in some individuals, testes under apoptosis were reduced to very small masses (Fig. 4c) in respect to the control shrimps (Fig. 4a, d). Clear signals of apoptosis in the *vasa deferentia* and in the AG’s of various individuals were detected by TUNEL under a UV complanar microscope (Fig. 5c). The acinous shape and the position of the AG gland yielded by the fluorescent dye was in accordance with the morphology of the gland observed in histological sections (Fig. 5d). The results of TUNEL tests (Fig. 5) were confirmed by TEM analyses (Fig. 6): thin sections showed DNA fragmentation and apoptosomes (dark vesicles containing nuclear materials and organelles) both in the male gonad (Fig. 6b) and in the androgenic glands. After pooling the results of all experimental sets, apoptosis was detected in male gonads within the treatments with *C. scutellum scutellum*, *C. scutellum parva*, *Diploneis* sp. and *C. neothumensis*, from the 5th to the 12th day of experiment (Table 2). In addition, we observed apoptosis of the AG in *Diploneis* sp. treatments, at the 7th day of experiment. Individuals sampled prior the 5th day and after the 12th day of experiment did not show any TUNEL fluorescence. Controls, as well, did not exhibit any TUNEL positive structure, from the 2nd to the 24th day of experiment.

**Discussion**

The number of larvae produced by each female and the growth rates obtained are in agreement with previous studies on *Hippolyte inermis* (Regnault 1969; Zupo 2000). The mortality rates were low enough to suggest absence of stress in the cultured shrimps. This point, in fact, may be critical for avoiding biases in the sex ratio due to stress (Austin and Meewan 1999), because recent studies indicated that stress may be one of the

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**Fig. 4** Confocal microscopy analyses (bars: 150 μm).

- **a** Positive control individual stained with 1,1-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine die, to show the shape, the position and the normal size of the main organs; **b** *Cocconeis scutellum parva* treatment at the 5th day of experiment. Male individual stained by the TUNEL technique; **c** *Diploneis* sp. treatment at the 7th day of experiment. Male individual stained by the TUNEL technique. **d** Negative control male individual fed on diet “M” stained by the TUNEL technique to show the absence of fluorescence. **o** optical chain, **s** gut, **g** gonad, **h** Heart, **d** vasa deferens, **a** Androgenic Gland, **j** gastrolites.
factors triggering the shift to female sex in protandric decapods (Bauer 2000; Calado et al. 2005).

The size reached by most shrimps at the end of feeding experiments was enough to guarantee a large percentage of mature individuals (Zupo 1994). Larger abundances of immatures, exhibited by the treatments with Amphora sp. and other diatoms, may be due to feeding deficiencies: *H. inermis* needs both animal and plant prey during the postlarval growth (Zupo 2001) and experimental diets may lack some feeding items (e.g. vitamins, fatty acids or essential aminoacids) indispensable to promote growth and maturation. However, the female/mature ratios were low, as expected (Zupo 2000), even in control treatments, indicating that sex ratios were not altered by any stress due to feeding deficiencies. Similarly, the slopes of growth

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**Fig. 5**  
**a** TUNEL detection of apoptosis in a 6-day-old male subjected to control treatment (diet “M”) observed under a UV complanar microscope (14×; bar: 500 μm) to show absence of apoptosis (i.e. absence of fluoresence).  
**b** 6-day old male under *Cocconeis neothumensis* treatment observed under a UV complanar microscope (25×; bar: 500 μm) to show absence of fluorescence (pre-treatment control). The rectangle on the bottom roughly corresponds to the area shown in (c), after the TUNEL treatment.  
**c** TUNEL detection of apoptosis in a 6-day-old male (the same shown in (b), prior to be treated) under *Cocconeis neothumensis* treatment, observed under a UV complanar microscope (230×; bar: 100 μm). The green fluorescence of tissues indicates the presence of apoptosis.  
**d** AG of a young male in a section stained by hematoxin-eosin, observed under an optical microscope (400×; bar: 25 μm).  
*a* Androgenic Gland,  
*d* deferens vasa,  
*g* testis

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**Fig. 6**  
**a** TEM image (2800×; bar: 5 μm) obtained on a transverse thin section of a testicular lobule from an individual of *H. inermis* subjected to a control diet, to show the absence of apoptotic processes.  
**b** TEM image (2800×; bar: 5 μm) obtained on a transverse thin section of a testicular lobule from an individual of *H. inermis* subjected to *Cocconeis neothumensis* diet, to confirm apoptotic processes and DNA fragmentation (arrow).  
*Ab* apoptosomes,  
n nucleus
Table 2  TUNEL positive structures detected on various days of treatment

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Days refer to postlarval growth (from 25 days after hatching, i.e. day 1, to 45 days after hatching, i.e. day 20). In most treatment groups, all days of culture were covered by collections, by sampling alternatively in different replicates, but only the days in which positive structure were detected are reported in the table. Empty cells indicate absence of fluorescence in any body region. “mg” indicates apoptosis of the male gonad; “AG” indicates apoptosis of the Androgenic Gland. The first row indicates the treatments, according to the acronyms reported in Table 1.

curves exhibited by most treatments were scarcely influenced by the composition of individual diets. In fact, differences among slopes were significant only for *Amphora* sp. and dry food (needing 30 days to reach the postlarval stage), as a confirmation of the lower efficiency of these diets. However, the growth efficiency descriptors (e.g. size, % immatures and % mortality at the end of the experiment) were not correlated in any way to the sex ratios. This evidence indicates that the factors influencing the growth rates are independent from the factors triggering the appearance of primary females in *H. inermis* populations (Calado et al. 2005).

It is worth observing that *Cocconeis neothumensis*, which previously demonstrated to effectively promote significant modifications of the sex ratio in *H. inermis* (large production of beta females; Zupo 2000), was one of the most effective diatoms, among the tested species. This study, in addition, demonstrated that *C. neothumensis* is not the unique diatom exhibiting an influence on the sex reversal of this shrimp (Zupo 2000), because other congenic diatoms (e.g. *C. scutellum parva*) promoted a similar effect, as well as different diatom genera (e.g. *Diploneis* sp.). Not all the considered diatoms triggered the production of primary females, because within the same species, the two subspecies *C. scutellum scutellum* and *C. scutellum parva* exhibited contrasting effects. Finally, such genera of benthic diatoms as *Amphora* sp. demonstrated negligible effects, as well as *Navicula* sp. and dry food (controls). We may conclude, therefore, that the factor produced by benthic diatoms, triggering the appearance of primary females in *H. inermis*, is unevenly distributed among various species and absent in several species of marine microalgae.

*Cocconeis sp.* are particularly abundant in the field (and in gut contents of *H. inermis*; Zupo 2001) in spring. This explains why the April offspring contains both males and small (primary) females (Zupo 1994). In contrast, these diatoms are scarcely abundant in fall (both in the field and in *H. inermis* gut contents; Zupo 2001). This explains why the fall offspring contains mainly males, which change their sex in the course of the next year (Zupo 1994). The results of molecular and ultrastructural analyses demonstrated that the influence of some diatoms on the sex reversal of shrimps is due to apoptosis (Vaux and Korsmeyer 1999), as hypothesized in previous studies (Zupo 2000). In fact, *H. inermis* lacks gonadic buds (Reverberi 1950) and the female gonad is produced starting from undifferentiated cells, only after the complete disruption of the testis. Hormonal influences of the ovary on the testis (e.g. an AG suppressor; Ginsburger-Vogel and Charniaux-Cotton 1982; Gherardi and Calloni 1993; Martin et al. 1999) may be excluded since an ovotestis was never observed (Reverberi 1950; Cobos et al. 2005). Therefore, the development of an ovary starts only after the suicide of the testis tissues. The discovery of the apoptotic disruption of the male gonad, selectively triggered by benthic diatoms, is important because it opens new biotechnological frontiers in the application of this fundamental mechanism of cell death (Hannun 1997; Jimeno 2002). Most apoptotic compounds applied to human medicine (Kaufmann and Earnshaw 2000; Frankfurt and Krishan 2001) as well as physical influences inducing cell death (e.g. X-rays) are “generalist effectors”: they act on a large variety of cells, in any physiological state. In contrast, the factor present in the considered benthic diatoms is effective only on the male gonad (and the androgenic gland) and exclusively in a short period of their life, i.e. from the 5th to the 12th day of growth.

Noteworthy, various marine natural products induce growth arrest and apoptosis of human neoplastic cells in vitro and in vivo (Schwartsmann et al. 2001; Jimeno 2002; Dirsch et al. 2004) and demonstrated strong activity against cancer and lower toxicity if compared with traditional chemotherapeutic agents. Some examples are aplidin, a marine compound isolated from *Aplidium albicans*, and ecteinascidin-743 derived from *Ecteinascidia turbinata*. (Broggini et al. 2003; Erba et al. 2001; Schwartsmann et al. 2003). Therefore, further studies aimed at elucidating the structure and the mechanism of action of the factors present in benthic diatoms, whose effectiveness was demonstrated by this study, could be crucial to clarify the cellular mecha-
nisms triggering specificity of the apoptotic effect, when directed against selected cell populations (O’Gorman and Cotter, 2001).

Another important finding of this study is the detection of apoptosis in the androgenic glands of shrimps fec on Diplopterus sp. This finding could help to understand the mechanism of action of the diatom compounds (Nagamine et al. 1980; Martin et al. 1993; Hengartner 2000). The disruption of the AG seemingly precedes the apoptosis of testes. It was not observed in all tested shrimps due to the size of these fundamental glands (few cells), the consequent rapidity of the process (few days) and the intervals (2 days) chosen for sampling the shrimps analyzed by the TUNEL technique. The testes, in contrast, are characterized by larger size, may need several days to be destroyed, and are frequently positive to TUNEL in the treatments CSS, CSP, CS and D. Suicide of testis tissues was detected in various phases, starting from the rear portion, close to the heart, and proceeding towards the anterior part, close to the gut (see Payen 1973 for a description of the morphogenesis of these structures in a decapod crustacean). These observations may be explained considering the fact that in crustaceans the androgenic gland is the sole source of hormones responsible for sex differentiation (Payen 1983; Abdu et al. 2002), i.e. the commitment of an embryo to either the female or the male pathway (Charniaux-Cotton 1954; Adiyodi and Adiyodi 1970). In male crustaceans, unlike male vertebrates, the endocrine and gametogenic functions are clearly separated into two distinct organs, the AG and the testis, respectively (Ginsburger-Vogel and Charniaux-Cotton 1982; Charniaux-Cotton and Payen 1988). In fact, sex differentiation can be manipulated through the removal of the AG, without damaging the gonads. In M. rosenbergii, for example, AG removal from immature males resulted in sex reversal, with complete female differentiation. Similarly, AG implantations into immature females lead to the development of a male reproductive system (Sagi 1988). Sex-reversed M. rosenbergii were capable of mating with natural males producing offspring. Therefore, the early disruption of AG in H. inermis feeding on Cocconeis sp., followed by the apoptosis of testes in the next 5 days, should be considered as the starting phase of H. inermis sex reversal (Zupo 2000).

Further studies will clarify the chemical structure of the apoptotic factor whose action was detected in this study, and they will allow for biotechnological applications of the compound (Bongiorni and Pietra 1996) and for clarification of the factors influencing the specificity of apoptosis in animal cells (Evan and Littlewood 1998). This study, however, demonstrated that the peculiar reproductive strategy of Hippolyte inermis observed in the field (Zupo 1994, 2001) is due to apoptotic compounds, present in variable concentrations in several benthic diatoms and all abundant during the spring reproductive period of H. inermis (Mazzella and Buia 1989). They are able to trigger the suicide of cells in the AG and in the male gonad of these shrimps in an early phase of the postlarval growth, so permitting the development of a primary female.

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Experimental evidence of a sex reversal process in the shrimp

Hippolyte inermis

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Summary

Hippolyte inermis Leach 1815 is a shrimp which forms stable populations in seagrass meadows of the Mediterranean Sea and along the Atlantic coast of Spain. Investigations from the last century have demonstrated specimens experiencing a male stage prior to switching to females (i.e., protandric sex reversal). Further studies have demonstrated that not all females are derived from sex reversal: young females apparently deriving from direct differentiation are present in natural populations. In recent years some authors have claimed that the species is simply gonochoristic, mainly based on the absence of ovotestis development. In order to establish if the species is a peculiar hermaphrodite or a simple gonochoristic, H. inermis postlarvae were individually cultured in Petri dishes in a semi-closed system. Their exuviae were regularly collected, fixed and stained, to monitor the sex and the size of each individual over time. In addition, histological sections were examined and we observed an individual who lost its appendix masculina and developed an active ovary. In contrast, specimens that retained their appendix masculinae exhibited a male reproductive system. Fourteen such individuals who lost their appendix masculinae were observed throughout the experiment, suggesting a mechanism of sex reversal in decapod crustaceans in which an ovotestis may be absent during the transition to the opposite sex.

Key words: Crustacea, Decapoda, Hippolyte inermis, sex reversal, histology, development, maturation

Introduction

Male sexual differentiation is thought to be mediated in crustaceans by hormones secreted by the androgenic gland (AG) (Charniaux-Cotton, 1967). All previous investigations on decapod crustaceans have demonstrated that sexual differentiation depends exclusively on the presence/absence of AG hormones (Sagi et al., 1997). The germinative zone in some species is largely determined as testis tissue due to the presence of the androgenic hormone. A reduction in the hemolymph levels of the hormone can prevent the male determination at given periods during the life of an individual.
individual, which may lead to intersex individuals and to sex reversal due to an auto-differentiation of the ovary (Sagi and Afifla, 2005; Baez, 2006). Therefore, the gametogenesis in hermaphrodite and gonochoristic crustaceans appears to be controlled by the same physiological mechanism, i.e., the presence or absence of the androgenic hormone (Weeks et al., 2006).

As a matter of fact, sex reversal is common among decapod crustaceans (Bauer, 2000). In the past, generalization over genera and families has led to some confusions and errors: in some cases hermaphrodite species were considered gonochoristic and vice-versa (Bauer and VanHoy, 1996; King and Moffit, 1984; Yaldwyn, 1966). For example, in the absence of direct observations, several Hippolytidae shrimps are still considered hermaphrodites due to their similarity to clean hermaphroditic species (d’Udekem d’Acoz, 2002).

Within the multivariate and complex sexual strategy adopted by decapod crustaceans (Yaldwyn, 1966), which includes gonochorism, sequential and simultaneous hermaphroditism and even parthenogenesis (Vogt et al., 2004), *Hippolyte inermis* Leach 1815 is a puzzling case. This shrimp, living in *Posidonia oceanica* and other seagrass meadows (d’Udekem d’Acoz, 1996), was first studied by Reverberi (1950), who discovered a peculiar mechanism of sex reversal. Reverberi (1950) demonstrated that *H. inermis* is sex-reversed only after the complete disruption of the male gonad, and the ovary is developed from embryonic undifferentiated cells. This suggested mechanism contradicts data from all other known decapods, in which an ovotestis is produced by the same germinal tissues as the testis and, subsequently, the regression of testes must occur to obtain a functional female (Bauer and Holt, 1998).

Yaldwyn (1966) and Veillet et al. (1963) confirmed that the shrimp reversed its sex about 1 year after hatching. Zupo (1994) confirmed again the presence of sex reversed females, based on a population dynamic investigation, but for the first time demonstrated the presence of primary females or, at least, very small females developing simultaneously with the male spring cohort. These were named “beta” females, in contrast to the larger “alpha” females deriving from sex reversal at the age of 1 year. Diatoms (*Cocconeis* sp.) were found in the gut contents of specimens sampled in the period of the appearance of beta females (Zupo, 2001). The feeding of *Cocconeis neothumensis* by *H. inermis* post-larvae has been shown to trigger the production of large amounts of beta females, while individuals fed on control foods (without diatoms) produced mainly males (Zupo, 2000). Recently, Zupo and Messina (2007) also suggested that the diatoms of the genus *Cocconeis* induce apoptosis of AG in *H. inermis*, followed by the complete disruption of the testis. This remarkable process leads to the production of beta females and supports the peculiar mechanism suggested by Reverberi (1950). However, Cobos et al. (2005) suggested that *H. inermis* is a simple gonochoristic species, based mainly on two observations: (a) the absence of any ovotestis in their histological sections and (b) not detecting reduction in the size of the *appendix masculina*, as observed in other sex-reversing decapods.

The present research was conducted in order to compare the conflicting conclusions of studies by Reverberi (1950) vs. Cobos et al. (2005) regarding the process of sex differentiation in *H. inermis*. In particular, starting from the well demonstrated absence of an ovotestis (all previous studies are in agreement about this point) we aim at understanding if the lack of an intersex stage means, as concluded by Cobos et al. (2005) that the shrimp is gonochoristic, or it means that the shrimp undergoes a peculiar process of sex reversal, as stated by Reverberi (1950). Direct observation of growing individuals in the laboratory was performed following morphological changes in their exuvia and reproductive system. In addition, we conducted histological investigations in order to provide support that the external sex characteristics observed (presence/absence of *appendix masculina*) were consistent with the internal reproductive organs.

### Material and Methods

All experimental specimens were derived from laboratory hatching of eggs laid by gravid females of *H. inermis*. Twenty-eight gravid females were collected in Lacco Ameno d’Ischia (Gulf of Naples, Italy) by a plankton trawl that scraped the surface of *Posidonia oceanica* leaves. After the collection, females were placed individually in 2 l aerated vessels containing 1.5 l of filtered and UV sterilized seawater, reared in a thermostatic chamber (18°C) with a 12:12 h photoperiod. Most females spawned 1–3 days after the collection and were then released into the field. Larvae produced by each female were divided into groups of 80 individuals. Each group was placed in a 1 l aerated vessel (containing 800 ml of filtered seawater) in the same thermostatic chamber. They were fed with *Brachionus plicatilis* (4 ind/ml) and *Artemia salina* nauplia (4 ind/ml) enriched for 12 h with a plant integrator (Algamac). Every 2 days larvae were collected using a 400 µm mesh filter and the culture media was renewed. After 25 days all larvae settled. Postlarvae deriving from different mothers were pooled, to exclude any maternal influence on sex, and divided into replicates of 20 individuals. Nine replicates were cultured in 500 ml dishes containing 400 ml filtered
seawater and a dry food (BDF), composed of 33% (by weight) of dry Artemia salina enriched with PUFA (Super-Hi Food Corp.), 33% of pure dried Spirulina and 33% of “AZ” shrimp food (Tetra Corp.). This basic food was pressed into small (5 mg DW) pellets. One pellet was administered every day to each dish. Every 2 days postlarvae were collected using a plastic pipette; the dish bottom was washed and water was replaced.

The food administered allowed for rapid growth of the larvae and postlarvae, with postlarval mortality as low as 13.9% on the 39th day after settlement. Most postlarvae reached sexual maturity after 39 days. In total, 155 postlarvae were individually transferred into small dishes (6 cm diameter) covered with a net (mesh size 0.2 mm) and clustered into an 80 l glass tank filled with filtered seawater. The tank was aerated with air-stones and an external filter (Eheim Classic 2215) filled with perlon wool and activated charcoal. The water flowing out from the filter was sterilized by UV (15 W) lamp prior to returning into the tank. Every 2 days the net covering each dish was removed, exuviae produced were collected and fixed in a solution of ethanol and rose Bengal (200 mg/100 ml 70% alcohol), and another small pellet of dry food was added to the bottom prior to restoring the dish in the tank. Fifty percent of the water in the tank was replaced every 2 days with clean seawater. The experiment lasted 279 days and it was concluded when only eight individuals were still alive. During this period the highest mortalities (20% and 40%) were observed during the 4th and 7th month of growth, respectively, in correspondence with two technical failures of the filtering system, producing temporary deterioration of the water quality.

The exuviae collected and stained were examined using optical microscopy. A picture of each exuvia was obtained by a Leica Z16-APO photomicroscope, equipped with a computerized system of image analysis permitting the measurement of total length (TL, from the tip of the rostrum to the posterior medial notch of the telson). The second pleopods on the exuviae were separated and photographed in order to record any change of secondary sex characters (appendix masculina) and to track biometry during the culture of individual shrimps.

Finally, some individuals were collected before or after the sex change for histological preparation and observation, to confirm the presence of primary sexual characters in shrimps whose sex was determined based on the presence/absence of the appendix masculina. For this purpose, shrimps were fixed in a modified Carnoy solution, dehydrated and embedded in paraffin. Serial sections (5 µm each) were stained in haematoxylin and eosin for optical microscopy observations. These preparations, as explained above, were not aimed at the detection of intersex individuals, since the absence of ovotestes was demonstrated in H. inermis by previous authors (Reverberi, 1950; Cobos et al., 2005). Plots were obtained to check the relationships between the size of appendix masculina and the total length of shrimps (King and Moffit, 1984).

**Results**

Fourteen individuals exhibiting an appendix masculina at the start of the experiment changed their sex throughout the test period, transforming into females (Fig. 1). A good correlation ($R^2 = 0.82$) was found between the number of these alpha (sex reverted) females and the average size of the shrimps at each time (Fig. 1). In contrast, the appendix masculina did not change its relative shape during shrimp growth and its length exhibited a linear correlation ($R^2 = 0.47$) to shrimp total length (Fig. 2). The collection and staining of each exuvia produced by cultured shrimps (Fig. 3) allowed for easy monitoring of the total length and...
Fig. 3. Images of stained exuviae and pleopods from *H. inermis* during the development of a representative individual. Loss of *appendix masculina* was observed at the fourth recorded moult, third month of culture, at a size of 14.43 mm as presented in the enlarged pictures of the second pleopod (reported below). Exuvies were collected in the dates reported on top of the figure. The second row indicates sex and size (total length expressed in mm) J, juvenile; M, male; F, female.

**Discussion**

The present study and laboratory culture demonstrated that 14 males lost their *appendix masculina* during the experimental period and developed the external sexual character typical of females. We have also demonstrated that males, externally identified by means of their *appendix masculina*, contained mature testes and that a female, identified after the lost of the *appendix masculina*, contained a mature ovary. These observations lead to the conclusion that the species is capable of sex reversal from male to female (Veillet et al., 1963; Zupo, 2000).

However, the presence of beta females (apparently primary females) in the spring cohort was demonstrated by Zupo (2004). The case of these small females, which appear to be derived from direct differentiation (Wenner, 1972), remains to be explained in order to find out whether these females are also the result of an early sex reversal process (Zupo and Messina, 2007). If these are primary females, it may support the hypothesis that this species represents a case of partial protandry, as observed in other decapod crustaceans (Rudolph et al., 2007). However, it is known that other factors (e.g., stress and growth conditions; Rider et al., 2005) may influence the sex maturation of decapod crustaceans.

The sex reversal process in protandric decapods is supposed to be accompanied by a size reduction of the *appendix masculina* when individuals approach the transitional period (King and Moffit, 1984; Schatte and Saborowski, 2006). Contrary to this, the biometric data found in the present study indicated that the *appendix masculina* increases its size in correlation with the total length of individuals and seems to be lost in a single moult when the sex reversal is recorded. This is in contrast with the observations made on most protandric decapods, but is in agreement with the data presented by Reverberi (1950). He suggested that the process is very fast and could lead to unparallel development of primary and secondary characters. This process is supported by his report on cases, in natural populations, of apparent males (individuals bearing a normal *appendix masculina*) containing an ovary while the male appendix is still present. Additionally, a small percentage of
Fig. 4. Dorsoventral sections of a representative male *H. inermis* showing the major features of its reproductive system. A, B, Dorsal section view. C, D, Mid-body section view. E, Ventral section view. F, High magnification of the terminal sperm duct showing the suspected androgenic gland tissue. Ts, testis; SD, vas deferens; AG, androgenic gland; WL, walking leg bases (indicated from the 1st to the 5th).
apparent females (individuals not bearing an *appendix masculina*) may be found, still containing a functional testis (Reverberi, 1950). Support to this notion comes from one such transitional male, found during the present research (Zupo, personal observation; data not shown).

The rapid sex change observed in these shrimps might also be explained according to the sex allocation theory (Charnov, 1982). The shrimp has only two narrow periods of reproduction every year, in April and September (Zupo, 1994), so it may be beneficial to the species when sex changes as quickly as possible, rather than progressing through several intermediate stages. This strategy could also explain the absence of an ovotestis, in terms of best allocation of resources (Speakman, 2005; Charnov et al., 2007). Moreover, the two periods of reproduction, in spring and fall, correspond to very different environmental conditions in terms of leaf density (exposure to predators), meadow spatial complexity (Zupo et al., 2006) and food availability. It is also known that sex ratios are differently controlled in a spatially variable environment (Charnov et al., 1981). Finally, we know that sex allocation may be size-dependent, with smaller hermaphrodites allocating more resources to male reproduction than larger ones (Baeza, 2007). The peculiar life strategy of *H. inermis* (double period of reproduction with different sexual strategy applied by young individuals and a variable proportion of resources invested in large and small females, respectively) may be viewed as a useful approach to increase mating opportunities and adjust sex allocations seasonally in order to improve its fitness to a high predation pressure (Zupo, 1994).

The morphometric evidence found, based on direct laboratory observations suggests that *H. inermis* is a protandric species, contradicting the conclusions reached by a previous study on the histomorphology of the female gonads collected from a field population (Cobos et al., 2005). The latter authors concluded that this species is gonochoristic based on: (a) the absence of ovotestis in their samples; (b) the absence of reduction in size of the *appendix masculina*, as expected for other protandric decapods; (c) the presence of small females in the natural populations. As stated above, the absence of an ovotestis in this species was previously observed by Reverberi (1950), whose evidence indicated that the sex reversal process does indeed occurring rapidly, during a single moult, and without an intermediate ovotestis phase. This critical stage should be studied more closely.

Bauer and VanHoy (1996) demonstrated that in some protandric species there is a reduction in the size of the *appendix masculina* as the individual approaches its sex reversal event. The absence of a reduction of the
appendix masculina prior to the sex reversal found by Cobos et al. (2005) is confirmed in the present study. In fact, in each of the 14 sex reverted individuals it could be seen that sex reversal occurred without any change in the size of appendix masculina.

The presence of smaller females, previously observed by Zupo (1994), could be the result of an early protandric sex reversal due to diatom food metabolites (Raniello et al., 2007). In fact, it was demonstrated (Zupo and Messina, 2007) that some diatoms induce quick destruction of the androgenic gland in H. inermis, which is the central controlling organ of male sex differentiation in crustaceans (Sagi and Khalaila, 2001; Sagi et al., 1997). The demonstrations of sex reversal reported above allow us to conclude that H. inermis is a protandric or, at least, partially protandric shrimp, in common with other decapod crustaceans (Rudolph et al., 2007).

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References


Abstract: Benthic diatoms of the genus Cocconeis contain a specific apoptogenic activity. It triggers a fast destruction of the androgenic gland in the early post-larval life of the marine shrimp Hippolyte inermis, leading to the generation of small females. Previous in vitro investigations demonstrated that crude extracts of these diatoms specifically activate a dose-dependent apoptotic process in human cancer cells (BT20 breast carcinoma) but not in human normal lymphocytes. Here, a bioassay-guided fractionation has been performed to detect the apoptogenic compound(s). Various HPLC separation systems were needed to isolate the active fractions, since the apoptogenic metabolite is highly active, present in low amounts and is masked by abundant but non-active cellular compounds. The activity is due to at least two compounds characterized by different polarities, a hydrophilic and a lipophilic fraction. We purified the lipophilic fraction, which led to the characterization of an active sub-fraction containing a highly lipophilic compound, whose molecular structure has not yet been identified, but is under investigation. The results point to the possible medical uses of the active compound. Once the molecular structure has been identified, the study and modulation of apoptotic processes in various types of cells will be possible.
1. Introduction

A variety of natural compounds with medical applications have been found in marine macro-algae [1,2], whereas the study of secondary metabolites from micro-algae and their biotechnological applications remains in its infancy [3,4]. Chemical ecology investigations may help find compounds with defined bioactivity [5,6] present in various diatoms, in particular those derived from the oxylipin pathway [7–9]. However, these studies are mostly related to planktonic organisms, often aimed at determining possible effects of diatoms on human health [10,11]. Very limited information is available about bioactive compounds produced by benthic diatoms and their biotechnological applications [12,13].

**Figure 1.** (a) *Cocconeis scutellum parva* observed under the SEM (7000×). (b) *Hippolyte inermis*, young male. (c) Sampling area for both the shrimp and the diatom (40°45′00″N 13°53′00″E), indicated by white arrows, at the Island of Ischia (right), in the bay of Naples, Italy (left).

The diatoms of the genus *Cocconeis* exhibit a peculiar biological activity effective on marine shrimps. In particular, *C. scutellum parva* (Figure 1a), seasonally present on the leaves of *Posidonia oceanica* and particularly abundant in spring, was demonstrated to induce an early shift to the female sex [14] in the shrimp *Hippolyte inermis* Leach (Figure 1b), living in the same environment. Still unknown compound(s) trigger a rapid apoptosis of the androgenic gland (AG) of this shrimp, followed
by sex reversal from male to female [15]. The strong influence of food on the sex of an invertebrate has been demonstrated only in this predator–prey relationship, and it is fundamental to assure stability to natural populations of this shrimp [16].

The proterandric sex reversal is a natural process in this species, occurring approximately after the first year of the shrimp life [17]. However, it has been shown [18] that the presence of ovigerous females would be continuously decreasing if based only on sex-reverted specimens (older than one year). In spring, the feeding on specific diatoms induces the production of early-developed females (small individuals, named Beta females) that contribute to the autumn reproductive season. Therefore, the apoptotic destruction of the AGs in males of H. inermis, triggered by the feeding on particular species of diatoms, represents a stabilizing factor for natural populations [14], since it increases the abundance of ovigerous females during the autumn reproductive season. This process is possible because the sex of crustaceans is determined by the presence/absence of a single gland, the AG [19], producing an insulin-like hormone that prompts the development of male characters [20]. The silencing of this hormone, prior to the appearance of male sexual characteristics, caused the full and functional sex reversal of males into neo-females, in the giant river prawn Macrobrachium rosenbergii [21]. Similarly, the ablation of the AGs induces a shift to the female sex in various crustaceans [22,23].

Specifically, in the case of H. inermis, the peculiarity of this process is in the selectivity of the action: A few species of Cocconeis diatoms [24] are active only on the small AG gland of this shrimp, in a very narrow time window (from the second to the twelfth day of post-larval life). The same species of diatoms were tested in other times [15] or on other decapods [25] but they did not elicit any bioactivity comparable to the one detected on young post-larvae of H. inermis. Additionally, toxicity tests performed with crude extracts of the diatom C. scutellum parva on embryos of the sea urchin Paracentrotus lividus did not elicit any significant effects [26]. Consequently, it may be worth identifying the metabolite responsible for the peculiar activity of these diatoms [27], characterized by a highly selective apoptogenic power, in order to develop new natural drugs useful for human anticancer therapies [6].

Programmed cell death (apoptosis) influences several biological processes in multi-cellular organisms [28], and it is involved in various diseases, including cancer [29]. The lack of activation of apoptosis leads to the proliferation of neoplastic cells in several organisms and most anti-cancer drugs currently used trigger the apoptosis of various types of cells [30], although their main disadvantage is represented by the wide spectrum of tissues targeted in the recipient body [31].

Previous research [32] demonstrated that apoptosis is triggered in human breast cancer cells (BT20 cells), in vitro, by the administration of very low doses of lipophilic fractions of the diatom C. scutellum parva, through the activation of caspase-8 and caspase-3, the initiator proteases involved in the extrinsic pathway, blocking the progression of the cell cycle from S to G2-M phases [33]. In contrast, the intrinsic pathway, mediated by caspase-9 [34], is not activated by C. scutellum extracts in cultured cancer cells. This demonstrates that the activation promoted by the diatom is not due to a generic toxicity [35], but it is due to the specific activation of cell surface apoptosis-inducing ligands [36].

Several marine algae have been demonstrated to produce apoptogenic compounds that could be promising candidate drugs for cancer therapy [37]. However, all of them exhibit a generic wide
spectrum of activity, inducing damage also in normal cells [38]. For this reason, the research of the specific apoptogenic compound contained in crude extracts of *C. scutellum parva* is promising.

Since the shrimp is so far the only biological “sensor” able to track the presence of the active compound(s), each new fraction obtained has to be tested on the shrimp’s larvae in order to detect the narrowest fraction containing the active compound, able to trigger the selective destruction of its AG. The research is complex because the compound is active at very low concentrations (0.1–10 ng of the extracted diatom biomass/mL), is likely present in very small amounts in the wounded cells, and is masked, as found during the HPLC analyses, by several other lipophilic compounds present in larger abundance in diatom cells.

In addition, the shrimp exhibits a single reproductive period every year, with two main peaks of recruitment [39]. Therefore, the production of new fractions and bioassays on shrimp post-larvae must be conducted yearly, since the research is limited by the field availability of ovigerous females. We report here the results of six years of bioassay-guided investigations, leading to the purification and characterisation of the active fraction produced by diatoms collected in Ischia (Italy; Figure 1c).

2. Results

2.1. Fractionation and Bioassays

Previous metabolite profiling [40] and VOC analysis [41] of *C. scutellum parva* demonstrated a large number of low-weight molecular compounds, though bioassays indispensable to trace the active fraction(s) were not conducted. In this study, the residue, composed of proteins, polymeric carbohydrates and silicate frustules, exhibited no activity when added to the food of *H. inermis* (Figures 1b and 2b). This evidence demonstrated that macromolecular compounds were not responsible for the induction of sex reversal. Therefore, the soluble part was separated by HPLC and divided into several fractions. These fractions covered the whole spectrum, from hydrophilic to extremely lipophilic components. The bioassays showed that two major activities were present, one among the hydrophilic compounds and another among the lipophilic compounds. Since in most experiments the lipophilic part was more active than the hydrophilic, we first characterized the lipophilic bioactive fraction and studied it in detail.

The first separation resulted in five fractions (Figure 2a). Fraction 3 was green and contained the highest amount of chlorophyll c and derivatives of chlorophyll a as indicated by the absorption spectra. The highest activity in terms of sex reversal was measured in fraction 4, with an F/mat% value of 53% ± 19%. Z-test indicated significant differences of this fraction, compared to the negative control (*p* < 0.01). The solid cell residue, in contrast, showed absence of activity. The bioassay indicated a skewed distribution of the activity in the fractions, with a minimum in fraction 1 and a maximum in fraction 4 (Figure 2b). For this reason, the next bioassay focused on further separations of fraction 4.
The main result of the second bioassay was the finding that the sub-fraction 4d (Figure 3a) exhibited the highest bioactivity of the lipophilic compounds (F/mat% 65 ± 15). An additional high activity was found in the hydrophilic part represented by fraction 2 (F/mat% 71 ± 20). Fraction 3, which contained the highest amount of chlorophylls, exhibited no activity. The solid residue was confirmed to be totally inactive (Figure 3b).
Figure 3. (a) Separation of *Cocconeis scutellum parva* extract performed in April 2009. HPLC chromatogram at 665 nm with the marked fractions; numbers in brackets indicate the year the separation was made. (b) Efficacy of the 7 fractions and of the residue indicated as percentage of females on the total of mature individuals (F/mat%). An asterisk indicates significant differences between treatments and the negative controls.

In the next separation, fractions 2 and 4 (exhibiting the highest bioactivity in former bioassays) were further separated into smaller sub-fractions (2a–d; 4a–d). The highest bioactivity of the third bioassay was again found in the fraction 4d (F/mat% 63 ± 1), which was nearly as high as the positive control (Figure 4a,b). The fractions 1 and 2a turned out to show medium bioactivity in the hydrophilic part of the chromatogram, with fraction 1 significantly higher than negative controls. Some fractions produced even a lower effect than the negative controls but the difference, in this case, was not significant (Z-test, p > 0.05).
Figure 4. (a) Separation of *Cocconeis scutellum parva* extract performed in September 2010. HPLC chromatogram at 665 nm with the marked fractions; numbers in brackets indicate the year the separation was made. (b) Efficacy of the 10 fractions and of the residue indicated as percentage of females on the total of mature individuals (F/mat%). An asterisk indicates significant differences between treatments and the negative controls.

The fourth bioassay was a repetition of the former separation and bioassay (Figure 5a). Again, fraction 4d (Figure 5b) turned out to have a bioactivity as high as the positive control (F/mat% 64 ± 1). A significant effect was produced by fraction 2c, as well. Also in this case some fractions exhibited an activity that was lower than negative controls, according to the natural variability inducing the presence of small amounts of females in any natural population.

Normally, the dried extracts were taken up in 100% methanol, and added to the food particles for the bioassays. However, since the activity could not be completely dissolved in methanol, we solved again the methanol-extracted residue in the vial of fraction 4d with acetonitrile (ACN). Considerable activity was found also in the second solution (fraction 4d ACN; F/mat% 50 ± 1; Figure 5b).
Figure 5. (a) Separation of *Cocconeis scutellum parva* extract performed in September 2011. HPLC chromatogram at 665 nm with the marked fractions; numbers in brackets indicate the year the separation was made. (b) Efficacy of the 10 fractions and of the ACN solved residue of fraction 4d, indicated as percentage of females on the total of mature individuals (F/mat%). An asterisk indicates significant differences between treatments and the negative controls.

2.2. Final Separation of the Bioactive Compound

The knowledge of the physical behaviour of the lipophilic bioactive fraction was used to introduce a new version of pre-concentration and to modify the HPLC separation. Such a pre-concentration is advantageous because higher amounts of the extracts can be administered to one HPLC separation. To allow diode array detection in the UV-C region, the separation was performed with isocratic conditions using solvents (acetonitrile and water) with low absorption in the range between 190 and 250 nm.

The diatom extract was subjected to a pre-separation on a C18 cartridge and eluted with 10% ACN (eluate A), 80% ACN (eluate B) and 100% ACN (eluate C), prior to analysis by HPLC (Figure 6a). The eluate C contained all the indicator absorption peaks in the lipophilic region (chromatogram at 195 nm). The bioassay confirmed the chromatographic data and showed the highest bioactivity in
eluate C (Figure 6b). In this case the three dried eluates were dissolved twice (as described in the experimental section), in methanol and then in acetonitrile, prior to their addition to experimental foods, and the bioassay indicated that the highest activity in the methanol-dissolved eluates is due to the eluate A (F/mat% 49.14 ± 18.69), while in the ACN-dissolved eluates it is due to the eluate C (F/mat% 48.28 ± 5.66). Both demonstrated significant differences towards the negative controls (Figure 6b), while eluate A dissolved in ACN exhibited an effect lower, but not significantly different in respect to negative controls.

Figure 6. (a) HPLC chromatograms (at 195 nm) of eluates A, B, and C of Cocconeis scutellum parva. (b) Efficacy of the C18 cartridge eluates indicated as percentage of females on the total of mature individuals (F/mat%) of the C18 cartridge eluates, after dissolving in methanol (MeOH) or acetonitrile (ACN), as demonstrated by the fifth bioassay. An asterisk indicates significant differences between treatments and the negative controls.
In the final separation, eluate C was used for further sub-fractionation of the lipophilic area (Figure 7a). After comparing fraction 4d obtained for the third and the fourth bioassays, under the same isocratic conditions as applied for the eluates A, B and C, the peak eluting at 19.8 min (which exhibited a maximum absorption at 197 nm) was chosen as a possible bioactive compound and collected separately (Figure 7a). In fact, both fractions 4d consistently showing high bioactivity exhibited no absorption in the range between 250 and 800 nm.

We therefore tested in the last bioassay five fractions derived by the eluate C. The bioassay demonstrated (Figure 7b) that fraction C3 (dissolved in acetonitrile in the last bioassay), consisting of this single peak, exhibited the highest activity on *H. inermis* (F/mat% 70 ± 12) and its activity was significantly different in respect to negative controls.

**Figure 7.** (a) Separation of eluate C in April 2013. HPLC chromatogram at 195 nm with the marked fractions of *Cocconeis scutellum parva*; numbers in brackets indicate the year the separation was made. (b) Efficacy of the five fractions and of the residue indicated as percentage of females on the total of mature individuals (F/mat%). An asterisk indicates significant differences between treatments and the negative controls.
3. Discussion

The bioactive lipophilic compound eluted from the HPLC column after the chlorophyll derivatives exhibited no absorption in the visible light spectrum. Applying isocratic low-light-absorbing solvents for separation, an absorption maximum of the active compound at 197 nm was observed by diode array detection. This evidence rules out compounds that contain functional groups with features of a chromophore. Such a conclusion is supported by the high lipophilicity of the compound. A high lipophilicity is also supported by the observation that MeOH is a much weaker solvent for the bioactive compound than ACN. Experiments have shown that the compound is very stable. Inactivation, due to storage or treatments, has not been observed. However, the ratios of activity between hydrophilic bioactive fraction and the lipophilic fraction varied in the batches tested. Therefore, we assume that hydrolytic processes may convert a hydrophilic cellular constituent by abstraction of a hydrophilic molecular part into a lipophilic compound. The lipophilic part would be responsible for the apoptotic activation while the hydrophilic part modulates the transport of the molecule. It is possible that such a reaction already takes place during the lyophilisation process or the subsequent extraction, as shown for the generation of free fatty acids [42]. However, also two structurally not related compounds could be present, since it was not yet shown that the isolated bioactive hydrophilic fraction may be converted into a bioactive lipophilic compound.

The bioassays following the first separation immediately indicated that fraction 4 contained a high activity and it could be a good candidate for further separations. The assays also demonstrated that the residue of the extraction, constituted by broken frustules and cell organelles, did not contain any apoptogenic activity. Therefore, the solvents used for the extraction and the techniques applied were effective and, since the second bioassay, it appeared clearer that two fractions were active, the first (fraction 2) more hydrophilic than the second (fraction 4d). This evidence might be due as well to a modification of a single compound during its bio-production process, which does not modify the primary active group. In addition, it was clear from the HPLC chromatograms that the apoptogenic compound could be present in small amounts and be masked by other compounds present in large abundance but with no bioactivity on the shrimp.

In fact, plots were dominated by chlorophylls and other pigments, peaking in the fraction 3, but also present in other fractions. All subsequent fractionations confirmed the positive results for fraction 4d, but the activity appeared to be shared by other fractions characterized by a higher polarity, in the range of fractions 1 and 2a. This activity was probably due to compounds characterized by narrow peaks in the fractions, which may be present in the last part of fraction 1 or in the first part of fraction 2a. Therefore, the compounds present in fraction 4d appear more stable and consistently positioned, while the biological activity of alternative compounds, present in the first eluates, is quite variable (maybe due to a variable level of esterification).

The presence of two compounds exhibiting comparable apoptogenic activity should not be a surprise since there are various apoptogenic compounds produced by marine diatoms [13], bearing a similar basic structure but being variously modified within diatom metabolic pathways [43], as, for example, galacto-pyranosyl-sn-glycerol, epoxy-eicosa-tetraenoic acid, hydroxyl-epoxy-eicosa-tetraenoic acid. In the latter case, for example, hydroxylation of a glycerol chain places compounds with different hydrophilicity but similar functions into groups [44].
Interestingly, when the vessel of the fraction 4d in the last bioassay was refilled with acetonitrile, an additional activity was detected. This demonstrates that the compound is highly lipophilic and that it is not totally soluble in methanol. The high activity exhibited both by fraction 4d in methanol and its further dissolution in acetonitrile also demonstrated that it is active at very low dosage: Even the residue compound still adherent to the vessel after the rinsing with methanol is able to trigger an evident biological activity. The active compound should not be a volatile organic compound (VOC) since these compounds, accurately investigated [41] in the same strain of C. scutellum parva, have been largely removed by repeated evaporations, according to the protocols used for the extraction and incorporation in experimental foods.

_Cocconeis scutellum parva_ contains a large number of mono-, di- and tri-unsaturated aldehydes with chain lengths from C$_5$ to C$_{10}$ [41] and some saturated aldehydes (heptanal, octanal, nonanal, decanal, undecanal), but it contains also fatty acid cleavage products of C$_5$ and C$_8$ chain lengths (aldehydes, vinyl alcohols and diones). Therefore, given the results of HPLC separations, the active compound might be a modified fatty acid (possibly in the most abundant categories of C$_5$ or C$_8$) with a peculiar functional group. Other investigations demonstrated the importance of the oxylipin pathways in various diatoms [13] and we know that polyunsaturated aldehydes (PUA) may be toxic and induce apoptosis and necrosis in the embryos deriving from females of marine copepods feeding on these microalgae [45]. In that case, however, the apoptosis was non-specific and it followed the destruction of various tissues as a result of their toxicity [46]. Our compound has a very specific apoptogenic activity and it was demonstrated to be non-toxic [26] when tested on sea urchin embryos. Therefore, our candidate compound might be related to the fatty acid pathway [47], be non-volatile [10] and non-toxic [48], and it could be characterized by a C$_5$ or C$_8$ chain length [41], probably involved in the mechanism of chemical defence of this diatom.

The chemical defences of marine algae are quite complex and highly evolved. Many of them have been related to invertebrate reproductive failures [13]. The ingestion of diatoms by copepods has been suggested as being detrimental for nauplii hatching success [49]. Also dinoflagellates inhibited the hatching success and larval survival of the scallop *Chlamys farreri* [50], and *Heterosigma carterae* was made responsible for suppressing the egg-hatching rates of the copepod *Acartia tonsa* [51]. In terms of reproductive impacts, the oxylipins are of great interest. They are produced in diatoms from fatty acids, mainly after wounding of the cells, and include (poly)unsaturated aldehydes, and hydroxyl-, keto-, and epoxyhydroxy fatty acid derivatives. They are known for their broad cytotoxicity that targets cytoskeleton, calcium signalling and cell death pathways [13,52]. Recently, two apoptosis-inducing galactolipids were isolated from the marine diatom *Phaeodactylum tricornutum* [36], and their apoptogenic effects were tested on mammalian cell lines. However, the apoptogenic effect from the as yet unknown compound of *Cocconeis* that leads to female sex is restricted to the androgenic gland, and is effective only in a short life stage of the shrimp.

In addition, it is rather unlikely that the apoptotic effects are caused by unsaturated straight chain aldehydes, because these molecules would have an absorption in the UV region and are volatile. This supports observations with planktonic marine diatoms that have a high capacity of aldehyde formation from polyunsaturated fatty acids but do not induce apoptotic reaction in the AG of *H. inermis* [53]. Thus, the bioactive principle must be different from the aldehydes that reduced egg-hatching rates, inhibited cleavage of sea urchin embryos, or arrested growth in CaCo2 cell lines [37].
In our case, although all bioassays confirmed the activity of fraction 4d, the presence of chlorophylls and other metabolites normally abundant in plant cells prevented an easy detection of the active compound that might have a promising apoptogenic activity. For this reason, a different fractionation method (Bond Elut C18, Varian) was applied in the fifth separation. In fact, this method allowed for pooling most plant metabolites in the first eluates, while maximizing the separation of other bioactive compounds. The fifth bioassay gave very clear results, indicating the compound was divided into two parts (as previously observed in the fourth bioassay), i.e., a relatively polar compound, present in eluate A, and a highly lipophilic compound maximizing into the eluate C. According to the bioassays 1–4, these two compounds could correspond to the ones separated into the fractions 1-2 and 4d, respectively.

It is also interesting to observe that the two mixtures of compounds are solved by different solvents: The activity of eluate A is at a maximum after solution in methanol, while the activity of eluate C is at its maximum in acetonitrile. This confirms the non-polar properties of the compounds present in eluate C, apparently corresponding to the ones previously separated in the fraction 4d.

For this reason, considering the higher stability and consistency of bio-activity of the fraction 4d in respect to more polar fractions, we decided to focus on the compounds present in this non-polar eluate, fractionating it into five parts (fractions C1–C5 in Figure 7b). The results are remarkable, indicating the peak of activity was determined by fraction C3. It likely contained a single compound (single UV-C peak), not corresponding to any standard already known. Thus, its structural elucidation is required.

4. Experimental Section

4.1. Diatom Isolation

Diatoms were collected by deploying metal panels painted with low-adhesion silicon coatings in the harbour of Ischia (Bay of Naples, Naples, Italy; Figure 1c) for 30 days, in April, when most species of Cocconeis produce natural blooms. Small amounts of micro-algae were scraped off the panels using a cover slide, and they were then cultivated in 3 mL plastic multi-wells in f/2 medium [54]. Diatoms were selected by means of a Leica micro-manipulator to obtain several mono-clonal cultures of Cocconeis scutellum parva. Mother cultures were then identified to the species level by SEM observations.

4.2. Diatom Culture and Harvest of Cocconeis Biomass

The cells growing on six mother-culture plastic dishes (4 cm) were collected by a Pasteur pipette, 15 days after inoculation, when the surface was almost completely covered by diatoms. The obtained diatoms were pooled in a sterilized beaker, and the suspension was divided into 20 parts and subsequently transferred to 20 Petri dishes (7 cm diameter) filled with f/2 medium. These dishes were kept in a thermostatic chamber for 15 days to obtain a diatom biofilm. After 15 days, the surfaces of the Petri dishes were covered with diatom biofilms, which were collected and pooled in a sterilized beaker. One mL of this suspension was inoculated into each of the 200 larger Petri dishes (14 cm diameter) containing the f/2 medium. The diatoms were cultured for 15 days in the same thermostatic chamber. After removal of the medium, the dishes were quickly rinsed twice with distilled water,
frozen at −20 °C and then lyophilized overnight. The dried diatoms were scraped off by a blade, weighed and kept in dry vessels at −20 °C until extraction. All the transfer operations were performed under a laminar flow hood and the dishes and laboratory instruments were previously autoclaved at 120 °C.

4.3. Extraction and Fractionation

The search for the bioactive compound was conducted during six subsequent years. This time was necessary because the shrimp’s spawning season allowed only one bioassay per year and each new production of diatom biomasses, for the subsequent extractions, required various months. After every bioassay-guided fractionation, with subsequent bioassay, HPLC separations were optimized based on the new information obtained, as reported in the following paragraphs and summarized in Table 1.

Table 1. Summary of the fractionation procedure followed during six subsequent years, indicating the biomass analysed, the separating procedure and the total number of fractions collected to perform subsequent bioassays, as reported in the figures of this paper.

<table>
<thead>
<tr>
<th>Sep. Nr.</th>
<th>Year</th>
<th>Biomass separated (by HPLC or C18 cartridge)</th>
<th>Extraction solvent</th>
<th>Separation procedure</th>
<th>Fractions collected</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2008</td>
<td>250 mg Cocconeis</td>
<td>80% ACN</td>
<td>HPLC; C18 analyt ¹</td>
<td>1-2-3-4-5 ³</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>2009</td>
<td>250 mg Cocconeis</td>
<td>80% ACN</td>
<td>HPLC; C18 analyt ¹</td>
<td>2-3-4a-4b-4c-4d-5 ³</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>2010</td>
<td>300 mg Cocconeis</td>
<td>80% ACN</td>
<td>HPLC; C18 analyt ¹</td>
<td>1-2a-2b-2c-2d-4a-4b-4c-4d-5 ³</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>2011</td>
<td>300 mg Cocconeis</td>
<td>80% ACN</td>
<td>HPLC; C18 analyt ¹</td>
<td>1-2a-2b-2c-2d-4a-4b-4c-4d-5 ³</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>2012</td>
<td>300 mg Cocconeis</td>
<td>Ca. 80% ACN</td>
<td>C18 Bond Elut Cartridge</td>
<td>Eluate A,B,C ³ ⁵</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>2013</td>
<td>560 mg Eluate C</td>
<td>80% ACN</td>
<td>HPLC; C18 analyt ²</td>
<td>C1-C2-C3-C4-C5 ⁶</td>
<td>7</td>
</tr>
</tbody>
</table>

1 linear gradient of 100% solvent A (80/20, v/v, MeOH/H₂O) to 100% solvent B (80/20, v/v, MeOH/acetone) in 10 min, then solvent B for 20 min; ² isocratic elution with 90% aqueous ACN; ³ fractions/eluates were dissolved in 100% MeOH and added to food particles for the bioassays; ⁴ fraction 4d was additionally reconstituted in ACN; ⁵ eluates were additionally reconstituted in ACN; ⁶ eluate C sub-fractions were dissolved in ACN only.

4.3.1. First Separation

Two hundred and fifty mg of freeze-dried biomass of C. scutellum parva were extracted with 12.5 mL aqueous 80% acetonitrile. The soluble part was separated from the insoluble by centrifugation (10 min) in Corex glass tubes at 7400 × g (DuPont Instruments, Newtown, CT, USA). The extract (equivalent to 200 mg of dry biomass) was separated into five fractions on an analytical C18 reversed phase HPLC column (GromSil ODS 4 HE, 250 × 4.6 mm, 5 µm particle size; Stagroma, Germany; 1 mL/min flow rate; 30 °C oven temperature; diode array detection) using a linear gradient of solvent A (80/20, v/v, MeOH/H₂O) and solvent B (80/20, v/v, MeOH/acetone). The time program was: Solvent A, 0% to 100% in 10 min, then solvent B, 100% for 20 min. The shift of the retention time of the eluted compounds between the first and the last separation was 0.1 min. The fractions were evaporated on a Rotavapor (Büchi, Switzerland) at 40 °C and 30 mbar. The residues were taken up in 5 mL 100% MeOH and transferred into glass vials. The solvent was evaporated in a nitrogen gas stream and the dry residues were stored at −20 °C. The equivalent of 200 mg Cocconeis extract were used in the bioassay.
4.3.2. Second Separation

An identical procedure, as above, was applied. In addition, since high bioactivity was found in fraction 4 previously tested, this was further divided into smaller fractions (4a–d).

4.3.3. Third Separation

At this stage we applied the same separation procedure as described above, but we increased the number of fractions, in order to determine more precisely the elution time of the bioactive compounds. In this case, we separated 300 mg of freeze-dried *C. scutellum parva*, and used 250 mg in the bioassays. Fraction 2 was further divided into four sub-fractions (2a–d), while fraction 3 was included into fraction 4a.

4.3.4. Fourth Separation

In this experiment the same separation and fractionation as reported above (third separation) was performed to verify the results of the previous year.

4.3.5. Fifth Separation

Three hundred mg of freeze-dried *C. scutellum parva* were gently potterized for 2 min in 6 mL of water (deionized ultra-pure water). The suspension was left at room temperature for 10 minutes, which allowed the action of wound-activated enzymes. After a transfer into a 50 mL centrifuge tube, acetonitrile was added in the ratio 1:10 (mL:mg) and incubated for 2 h in the dark. After centrifugation (4000 rpm, 10 min; Centrifuge Heraeus Christ GmpH, Hanau, Germany) at room temperature, the supernatant was collected for further separation on a cartridge (Varian Bond Elut C18, Agilent Technologies, Basel, Switzerland) applying slight vacuum. The cartridge was cleaned and equilibrated by using one volume (60 mL) of 30%, 50%, 80%, 100% and finally 30% aqueous ACN. The cartridge was never allowed to get dry during the passage. Ten mL of the diatom extract was combined with 16.6 mL H$_2$O, thoroughly mixed and passed through the equilibrated cartridge. Shortly before becoming dry, 33.4 mL of 30% aqueous ACN was added. Thus, eluate A had a final concentration of 10% ACN. In a second step, the cartridge was eluted with three volumes of 80% ACN (180 mL) to remove the green chlorophyll derivatives (eluate B). Finally, the cartridge was eluted with two volumes of 100% ACN (120 mL) to get the eluate C. The low-boiling organic solvent ACN was removed on a rotary evaporator Rotavapor R-200, Büchi (Newcastle, DE, USA), and subsequently the water was removed using a Liophilizator Lio5P, 5Pascal (Milan, Italy) until dry residues were obtained. The residues were dissolved in 5 mL ACN and transferred into smaller glass vials. After removal of the solvent (Rotavapor) the dried extracts were stored at −20 °C until further use. The equivalent of 300 mg *Cocconeis* extract were used in the bioassay.

4.3.6. Sixth Separation

Since the bioactive compound seemed to have no important absorption in the long-wave UV, we changed the solvents used for separation and applied isocratic conditions. Acetonitrile and water,
which allowed seeing UV-spectra in the UV-C range, were used for further analyses. Eluate C (ACN) was chosen for further analyses, according to the results of bioassays. Eluate C (equivalent to 560 mg freeze dried biomass of *C. scutellum parva*) was dissolved into 2 mL 80% aqueous acetonitrile. The extract was separated into five fractions on an analytical C18 reversed phase HPLC column GromSil ODS4 HE, Stagroma GmbH (Reinach, Switzerland) 250 × 4.6 mm; 5 µm particle size; 1 mL/min flow rate; 30 °C oven temperature; diode array detection). Solvent A was UV-treated H₂O, while solvent B was acetonitrile. All separations (20 runs) were done with 90% B under isocratic conditions. The peak (195 nm) eluting at 19.8 min was chosen as a possible bioactive candidate due to former separation procedures and it was collected into a single fraction. The equivalent of 300 mg biomass was tested in the bioassays.

4.4. Bioassay Procedure

For the bioassays we followed the techniques described by Zupo and Messina [15]. The seawater used for the experiments was filtered overnight through a mechanical absorbent filter 250 Classic, Eheim GmbH (Deizisau, Germany) containing perlon wool and activated charcoal. The water was treated with ozone for 4 h and aerated 4 h prior to be used for larval cultures.

Ovigerous females of *Hippolyte inermis* were collected in a *Posidonia oceanica* meadow off Lacco Ameno (Island of Ischia, Naples, Italy), sorted on boat and kept in plastic bags up to the return in the laboratory. The ovigerous females were then transferred individually into 2 L conical flasks containing about 1.8 L of filtered seawater, along with a small portion of *Posidonia* leaf, for shelter, in a thermostatc chamber maintained at 18 °C, with Gro-Lux fluorescent tubes at a mean irradiance of 250 µmol m⁻² s⁻¹ ten hours a day. After some days, each female released larvae (from 20 to 400) that were collected and kept for our bioassays. Their mothers were immediately returned to the sea. The larvae collected were transferred in groups of 80 in 1 L conical flasks containing approximately 800 mL of clean filtered seawater, kept in the same thermostatic chamber as described above.

Larvae were fed by adding four *Artemia salina* nauplii and four *Brachionus plicatilis* individuals per mL of seawater, for the first 7 days. During the following days, the administration of *Brachionus* was interrupted and *Artemia nauplii* were enriched using an Algamac, Biomarine (Hawthorne, CA, USA) integrator [15]. After about 30 days the larvae settled and they were transferred in groups of 25 post-larvae into 14 cm (diameter) vessels containing 400 mL of filtered seawater. They were fed a composed food made of three items in equal proportions: SHG “*Artemia* Enriched”, SHG “Microperle” and SHG “Pure Spirulina”. This is the composition of the basic food used for our negative controls. To test the activity of *C. scutellum parva*, its dried extracts were dissolved in 2 mL of methanol (MeOH) and mixed to the prepared composed food. The solvent was then evaporated by a rotary evaporator to obtain the dried foods supplemented with the diatom fractions.

To prepare the positive controls, dried diatoms (*C. scutellum parva* of identical batches) were added to the basic food in a ratio of 2:1 (w/w). All foods were prepared at the start of the experiment (the first day of post-larval growth) and kept in a refrigerator at −20 °C up to the administration. Dry food (5 mg) was offered daily in each vessel containing 25 post-larvae of *H. inermis*.

In detail, we assayed three replicates (25 individuals each) for each negative and positive control, as well as three replicates (25 individuals) for each fraction to be tested. The daily mortality was
measured by collecting and counting larvae by a Pasteur pipette, while water and food were replaced. Post-larvae were sacrificed after 40 days, fixed in 70% ethanol, and subsequently observed under the dissecting microscope to measure their total body length using millimetric paper and a metal bar. Their second pleopods were collected on a slide and analysed under the optical microscope to check the presence or absence of a masculine appendix, indicating male or female sex, respectively.

Each bioassay tested the activity of further fractions (as above reported) against positive and negative controls. In particular, the first bioassay tested the activity of five fractions obtained from the first separation. In addition, we measured the activity of the “residual”, consisting of frustules and broken diatoms remained in a pellet after the centrifugation of the extraction solvents. This test was aimed at checking if the chosen solvents effectively extracted all active compounds.

The second bioassay tested the activity of seven fractions obtained from the second separation, as reported previously. Also in this case the activity of the “residual” was checked in order to exclude that the active compound was still present in the solid pellet after centrifugation.

The third bioassay tested the activity of 10 fractions obtained from the third separation. The fourth bioassay tested again the activity of 10 fractions obtained from the fourth separation to confirm the data of the previous one. In addition, we measured the activity of the fraction 4d twice. The first test was performed by adding 2 mL of methanol (667 µL for three times) to the vessel containing the dried fraction 4d. It was vigorously shaken and added to the dry food. For the second test, the same vessel was refilled with 2 mL of acetonitrile (667 µL for three times) and the procedure was repeated. In this way, we aimed at checking if the solvent used to transfer the fractions to the experimental foods (methanol) was able to completely dissolve the active compound in the lipophilic fraction 4d.

The fifth bioassay was performed on three eluates obtained with a different separation method, as reported previously. In this case, all tests were repeated twice: The first time the eluates were dissolved and transferred to the dry foods using 2 mL of methanol. The second time, the same vessels were refilled with 2 mL of acetonitrile, then transferred to dry foods and evaporated. In this way we wanted to test the efficacy of methanol in dissolving completely the active compound present in the dry film covering the vessels.

The last bioassay was performed on five fractions of the eluate C (sixth separation), reconstituted in acetonitrile, as indicated above.

4.5. Statistical Analyses

The percentage of females normalized to the total number of mature individuals (F/mat%) was calculated for each test. This index permits to determine the effect of apoptogenic compounds on the sex of mature shrimps, avoiding the influence of individuals that, at the end of the experiment, were still immature. The significance of differences between each treatment and the controls was tested by applying the Z-Test on proportions (STATS 2002 v. 2.7) for the observed F/mat% values.

5. Conclusions

A strict bioassay-controlled separation was performed in the present investigation and led to the characterization of bioactive fractions. Freeze-dried biomasses of C. scutellum parva were extracted with 80% aqueous ACN, and all the activity was found in the extract. Currently, the structure
elucidation of the bioactive compound is in progress; however, time is needed to obtain a sufficient amount for spectroscopic analyses, such as HR-MS and NMR. Future research will test the activity of the pure compound on our model shrimp to confirm the identity of the apoptogenic compound. This confirmation, in fact, will disclose new challenges, since the comprehension of the mechanism of action on the cell machinery [55,56] could indicate the factors underlying the specificity of the apoptotic process [34], leading to further important medical applications.

Acknowledgments

Diatom collections were performed by Patrizia Messina with the technical help of Cpt. V. Rando. We are grateful to Mario De Stefano for the S.E.M. taxonomical identification of our mother cultures. Carmen Patalano cooperated in the laboratory cultivation of diatoms. C. Maibam and E. Butera were supported by a Stazione Zoologica grant, within their Open University PhD studies under the supervision of V. Zupo. Luigi Pizzo and Mirko Mutalipassi (Master thesis under the supervision of V. Zupo) cooperated in the shrimp culture during some bioassays. We are grateful to E. von Elert (Cologne University) for his critical revision of our manuscript and to R. Messina for the revision of English language.

Conflicts of Interest

The authors declare no conflict of interest.

References


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Chapter 3.6. Food is information: the role of wound-activated infochemicals

Summary

3.6.1. Odour compounds of the diatom Cocconeis scutellum: effects on benthic herbivores living on Posidonia oceanica. (Cited by 33. I.F.= 2.62)
Food is information and wound-activated infochemicals generate a web of communication among diatoms and benthic invertebrates. The effects have been identified through experiments on the chemotactic behaviour of invertebrates to detect the meaning of their evolutionarily evolved choices.

3.6.2. Ecological role of diatoms as regulators of invertebrate physiology and behaviour. (Cited by 6. I.F.= n.d.)
Diatoms play fundamental trophic roles both in the marine benthos and plankton. The knowledge about their regulatory influences on benthic invertebrates is discussed since they are demonstrated to be both physiologic regulators (through the food webs) and infochemicals perceived by chemosensory structures and triggering defined chemotactic reactions.

3.6.3. Chemoreception of the seagrass Posidonia oceanica by benthic invertebrates is altered by seawater acidification. (Cited by 12. I.F.= 2.45)
Food is energy and food is a physiology regulator. However food is also information. Several plants and invertebrates interact and communicate by means of volatile organic compounds (VOCs). These compounds play the role of infochemicals, being able to carry complex information to selected species, thus mediating inter- or intra-specific communications.
The VOCs have been extracted and tested on a set of 13 species of associated invertebrates to identify their specific chemotactic responses in order to determine if: a) seagrasses produce VOCs playing the role of infochemicals, and b) their effects can be altered by seawater pH. Results indicate that several invertebrates recognize the odour of wounded *P. oceanica* leaves, especially those strictly associated to the leaf stratum of the seagrass. Thus, leaf-produced infochemicals may influence the structure of *P. oceanica* epifaunal communities, and their effects can be regulated by seawater acidification.

3.6.4. **Relevance of wound-activated compounds produced by diatoms as toxins and infochemicals for benthic invertebrates.** *(Cited by 14. I.F.= 2.39)*

Plants evolved the production of toxic wound-activated compounds (WACs) to reduce grazing pressure. In addition, several plant-produced WACs are recognized by invertebrates, playing the role of infochemicals. The specific toxicity of WACs is inversely correlated to the perceptive ability of invertebrates towards volatile compounds liberated by the same algae. Hence, when the recognition of specific algae by a given invertebrate species evolves, their detrimental effects on the receiving organism may be lost.

3.6.5. **Centropages typicus** *(Crustacea, Copepoda) reacts to volatile compounds produced by planktonic algae.** *(Cited by 9. I.F.= 1.79)*

The ability of the copepod *Centropages typicus* to perceive the odour of three planktonic diatoms (*Skeletonema marinoi, Pseudonitzschia delicatissima* and *Chaetoceros affinis*) and a dinoflagellate (*Prorocentrum minimum*) was investigated. This information is ecologically relevant for orientation, habitat selection, predator avoidance and communication. In addition, as the pH of the medium influences the perception of chemical
cues in aquatic environments, the effect of seawater acidification resulting from increasing levels of CO$_2$ was tested, along with its influences on the olfactory reactions of copepods. Seawater acidification induces changes in copepods’ perception of odours. These findings highlight the sensitivity of chemically-mediated interactions to global changes.
Odour compounds of the diatom *Cocconeis scutellum*: effects on benthic herbivores living on *Posidonia oceanica*

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ABSTRACT: Polyunsaturated aldehydes released by marine planktonic diatoms upon wounding have been extensively studied since the discovery that they reduce the hatching success of copepods. Diatoms are also abundant in benthic marine ecosystems. For this reason we investigated the presence and the possible ecological roles of *Cocconeis scutellum parva*, a diatom found tightly attached to the leaves of *Posidonia oceanica*. This diatom was previously demonstrated to play an important trophic role for the shrimp *Hippolyte inermis*, by influencing its sex reversal. In the present study, *C. scutellum parva* was isolated and cultivated and the volatile compounds released upon cell disintegration were identified by mass-spectrometric methods. We demonstrated the presence of unsaturated aldehydes with chain lengths from C₅ to C₁₀. Unexpectedly, we found C₆ compounds represented by 3(Z)-hexenal, 2(E)-hexenal, hexanal and hexanol-1 that resemble the typical bouquet observed in higher plants. Compounds not described before for diatoms were, among others, pentane-2,3-dione and octane-2,3-dione. Food choice experiments performed on 17 animal species associated with *P. oceanica* meadows indicated that these grazers recognise the presence of the odour compounds, exhibiting complex patterns of reactions according to their life strategies. The mosaic of results obtained at various concentrations, in different species, indicated that wound-activated infochemicals generate a web of communication among diatoms and benthic invertebrates.

KEY WORDS: Infochemical · Odour · Seagrass · Behaviour · Feeding · Wounding · Diatom

INTRODUCTION

*Posidonia oceanica* (L.) Delile is a Mediterranean seagrass characterised by high stability of associated plant and animal populations, long persistence and great ecological importance (Buia et al. 1992), since its extensive and dense coastal meadows are considered to be biodiversity hotspots (Orth et al. 2006). A complex web of trophic, physiological, spatial and chemical relationships is produced by the stable coexistence of associated plant (Michael et al. 2008) and animal (Buia et al. 2000) populations. The relationships between *Hippolyte inermis* Leach and its diatom food are an excellent example. *H. inermis* is a shrimp that lives in the leaf stratum of *P. oceanica*, whose periphyton is often diatom-dominated (De Stefano et al. 2000). *H. inermis* is considered an opportunistic herbivore, grazing on algae present on the leaves of *P. oceanica* and, therefore, ingesting large amounts of epiphytic diatoms (Zupo 2001). We demonstrated that the ingestion of diatoms ascribed to the genus *Cocconeis* (in particular *C. neothumensis*, *C. scutellum parva* and *C. scutellum scutellum*) triggers the apoptosis of its androgenic gland (Zupo & Messina 2007), followed by the complete destruction of the male gonad and a shift to the female sex (Zupo 2000). This effect was demonstrated to be positive for natural populations since the production of young females in spring (when the abundance of *Cocconeis* spp. diatoms on the leaf stratum is very high) assures higher reproductive bursts (Zupo 1994). Therefore, in *H. inermis*, the ingestion of diatoms is helpful for natural populations and the pro-apoptotic
compound (whose structure has not yet been identified; Nappo et al. 2009) present in these diatoms acts as a spring signal for the production of young females, probably due to a long co-evolutionary process (Zupo et al. 2007).

In other cases, secondary metabolites produce deleterious effects on consumers. Compounds responsible for the biological effects have been demonstrated to be volatile organic compounds (VOC) not present in intact cells but released by a lipoxygenase cascade upon cell wounding (Pohnert 2000, Jüttner 2005). Unsaturated aldehydes of the lipoxygenase cascade in planktonic diatoms exhibit teratogenic effects on copepod larvae and reduce the viability of their eggs (Miralto et al. 1999). Wound-activated diatom cells also release unsaturated aldehydes (Pohnert 2002) that are inhibitors of mitotic proliferation in sea urchin embryos (Miralto et al. 1999). Similar relationships, although less striking, were demonstrated between the crustacean Daphnia pulex and some freshwater diatoms (Carotenuto et al. 2005).

Secondary metabolites produced by the lipoxygenase pathway may also act as repellents against grazers (Fink et al. 2006). The lipoxygenase product 2(E), 4(E),7(Z)-decatrienal has been shown to act as a repellent for freshwater crustacean grazers (Jüttner 2005). Therefore, it is evident that diatoms producing volatile compounds have a range of possible consequences. Co-evolutionary processes may modulate their effects, as is the case of VOCs that have been shown to play a role in locating suitable habitats for aquatic insects (Evans 1982) and nematodes (Höckelmann & Jüttner 2004). All this evidence indicates the presence of a complex network of relationships shaped by diatom infochemicals. The data published in recent years on this topic have spurred interest and several studies indicate benthic invertebrates can detect and differentiate VOCs released from diatoms (Jüttner & Dürst 1997, Jüttner 2005) and utilise them as food-finding cues. In these cases, the diatom-produced infochemicals can be classified as foraging kairomones (Ruther et al. 2002).

Based on these assumptions, we may also ask if Cocconeis spp. diatoms, which are present in and seasonally dominate; De Stefano et al. 2000) the leaf stratum of Posidonia oceanica with known effects on the physiology of selected shrimps (Zupo 2000), produce VOCs and if their ‘odours’ may act as food-finding cues for some of the typical animals associated with the leaf stratum of the seagrass. In fact, P. oceanica represents a nest environment for several invertebrates and some fish, especially in spring, which is also the season of maximum abundance of Cocconeis spp. in the leaf stratum (Mazzella & Buia 1989, De Stefano et al. 2000). To answer this question, we analysed the VOC composition of C. scutellum parva cultured in the laboratory, re-constructed its VOC bouquet from pure compounds and performed binary-choice experiments to test its effect on selected invertebrates and on juveniles of one fish species.

**MATERIALS AND METHODS**

**Collection and culturing of epiphytic diatoms.** Collecting and culturing Cocconeis scutellum parva is complex, since this diatom is very adhesive, slow-growing and delicate. Therefore, the classical collection and selection methods (Chepurnov et al. 2004) used for other species of benthic or planktonic diatoms are not applicable. For isolation of Cocconeis spp., metal panels (30 cm × 40 cm) were coated with a low adhesion silicon polymer (Terlizzi et al. 2000) and deployed in the harbour of Ischia (Gulf of Naples) at 2 m depth in April 2001. After 1 mo of exposure, panels were gently sprayed with seawater to remove foulers. Most Cocconeis spp. diatoms, being the most adherent epiphytes, were conserved on the surface. Several small samples of diatoms were obtained by scraping about 1 cm² of the surface with the aid of a coverslip. The collected samples were individually suspended in Petri dishes containing 3 ml of sterilised seawater. Cocconeis spp. diatoms were identified at the genus level by inverse optical microscopy, collected by means of a glass capillary tube connected to a micromanipulator (Leica Micromanipulator M) and individually transferred to multiwell dishes (Falcon™ 35-3046, 6 wells of 5 ml) containing sterilised seawater. This operation was repeated several times, in order to separate the diatoms from bacteria, spores, cyanobacteria and other organisms (see Zupo & Messina 2007 for details). The wells were examined every 3 d in order to monitor the growth of Cocconeis spp. When small clusters of diatoms were obtained, newly produced diatoms were transferred again, using a glass capillary tube connected to the micromanipulator, and cultured in sterilised f/2 Guillard’s medium (Sigma G9903). We considered the diatom culture sufficiently clean after 3 transfers to new wells, and proceeded to species identification using scanning electron microscopy. Diatoms were subsequently scraped off using a sterilised Pasteur pipette, washed in distilled water and collected on a Millipore filter. The filter was then glued onto a microscopy stub and gold-sputtered prior to the observation. We were thus able to isolate 32 strains representing 5 species of epiphytic diatoms (C. neothenensis, C. scutellum scutellum, C. scutellum parva, C. posidoniae and Diploneis sp.). All these diatoms were conserved at the Benthic Ecology Laboratory of the Stazione Zoologica Anton Dohrn (Naples, Italy) in
thermostatic chambers until used in experiments conducted in July 2008. Replicate cultures of each strain were maintained in different chambers, in f/2 medium at 18°C, under a 12:12 h photoperiod and irradiance of about 150 µmol m⁻² s⁻¹. Diatoms were transferred to new Petri dishes every 15 d and inspected monthly by inverse optical microscopy.

**Preparation of diatoms for extractions.** Six Petri dishes containing pure cultures of *Cocconeis scutellum parva* were completely scraped off using a sterilised Pasteur pipette and their content was pooled in a glass vessel. This suspension was used to prepare several cultures in 7 cm Petri dishes containing f/2 medium. When diatoms covered the bottom of the Petri dishes, they were collected, and the suspension obtained was used to inoculate 20 large (14 cm diameter) plastic Petri dishes containing f/2 medium. The large Petri dishes were then cultivated in a thermostatic chamber for a further 15 d. The growth medium of each Petri dish was then removed and the dishes were frozen at –20°C.

**Counts and cell volumes.** The cells on the bottom of each Petri dish were counted under the inverse optical microscope. For this purpose, 20 fields were randomly chosen and examined; the diatoms present in each field were counted; finally, a proportion of the whole area of the plate was applied to calculate the average number of cells per plate. In addition, the average cell volume was calculated based on observations made on a single plate. For this purpose, 50 randomly chosen diatoms present on the plate were measured (length and width). The thickness of diatoms was impossible to evaluate in this way, since *Cocconeis* spp. cells are very thin and normally attached to the bottom. Therefore, 50 diatoms from the same strain were measured under the scanning electron microscope and an average thickness of 2.31 µm was obtained. This thickness was then used for the calculation of the cell volume based on the formula (Hillebrand et al. 1999, Sun & Liu 2003):

\[
\text{Volume} = \pi / 4 \times \text{length} \times \text{width} \times \text{thickness}
\]

**Activation of Cocconeis scutellum parva.** Petri dishes (14 cm diameter) with a dense layer of *C. scutellum parva* patches were kept frozen at –20°C. Analyses were carried out within 8 d after freezing. A Petri dish was taken out of the freezer and 5 ml of brine (20% NaCl in water, purified twice by stripping) was poured over the cell lawn. The cells of *C. scutellum parva* were physiologically disintegrated leading to the activation of lipoxygenase cascades and the formation of volatile compounds (Jüttner 2005). This was also indicated by the appearance of a strong rancid odour. The cells were detached with a rubber spatula and the suspension transferred into a round bottom flask of glass for stripping analysis. The time needed for this procedure was about 10 min and was sufficient to allow the production of VOCs.

**Stripping analysis.** The VOCs were concentrated by closed-loop stripping and analysed using gas chromatography-electron impact mass spectrometry (GC-EIMS) (Durrer et al. 1999), but the Erlenmayer flask was replaced by a 250 ml round-bottom flask. Brine was used to detach the cells on the Petri dish and to quantitatively transfer the suspension into the glass vessel. The stripping time was 45 min at 22°C, after which most of the VOCs were adsorbed on Tenax TA. The Tenax-filled cartridge was removed, and the VOCs were thermally desorbed and transferred into a GC-EIMS as previously described (Jüttner 2005).

**Identification of compounds.** For identification of the VOCs, chromatograms of reference compounds that exhibited similar concentration as the cellular compounds were established. When the retention times and mass fragmentation patterns were identical for both the reference and the *Cocconeis scutellum parva* constituent, a compound was considered identified. Mass spectrometry (MS) data from the literature, the National Institute of Standards and Technology (NIST) library and our own library were used for preliminary identification.

**Reference compounds.** If not otherwise stated, the reference compounds were purchased from Fluka. 2(4Z,6E)-Nonadienal was from Roth. Unsaturated aldehydes, ketones (3,5-octadien-2-one) and alcohols (3,5-octadien-1-ol) of the C₈ skeleton were obtained from fruiting bodies of commercial *Agaricus bisporus* (Tressl et al. 1982). Part of the brown mushroom cap was ground in an agate mortar, transferred with 50 ml of water into a round-bottom flask and treated as above (see ‘Stripping analysis’) for stripping and gas chromatography-mass spectrometry (GC-EIMS) analysis. 1-Hexen-3-one was prepared from 10 µl 1-hexen-3-ol in toluene by oxidation with MnO₂ (2 h at 50°C). 2(E),4(6(Z))-Decatrienal was a synthetic compound (Wendel & Jüttner 1996). Octane-2,3-dione was donated by Givaudan Schweiz.

**Quantitative determination.** *Cocconeis scutellum parva* was grown in Petri dishes because solid substrate was an essential prerequisite for cell growth. After decanting the medium, the wet biomass that was still attached to the Petri dish was frozen. The concentration per cell was calculated using an independent determination of the VOC of 1 and of 4 Petri dishes. Thawing and the addition of 20% NaCl solution activated several lipoxygenase reaction cascades, and several volatile compounds were released. These odour compounds were concentrated by closed-loop stripping and analysed by GC-EIMS. The VOCs were determined using the integrated areas of single ions that were
extracted from the total ion chromatograms. The following features were taken into account in the selection of ions: sufficient intensity, specificity for a particular compound and low contamination by other unresolved compounds. When the peak was contaminated by another compound, the uncontaminated half of the peak area was determined and doubled assuming peak symmetry. The calibration (3 replicates) was done with reference compounds under the same conditions as the samples. When reference compounds were not available in sufficient purity to allow calibration, total ion currents (in the ranges m/z 33 to m/z M+1) were used, and calibration was performed with structurally related compounds. 2(E)-Hexenal was used to calculate 3(Z)-hexenal and 1-hexen-3-one, 1-octen-3-ol to calculate 2,5-octadien-1-ol, and 2(E)-octenal to calculate 3,5-octadien-2-one and 2(E)-nonenal.

**Odour choice experiments.** The experimental design consisted of 6 replicates of 5 individuals each, for 17 animal species associated with *Posidonia oceanica* meadows (Table 1). The specimens necessary for the experiment were collected in Lacco Ameno d’Ischia (Gulf of Naples) by a circular net (1 m diameter; 300 µm mesh), trawled on the surface of *P. oceanica* leaves, at a depth between 5 and 12 m. After collection, the fauna was sorted into 17 species (considering only those for which at least 40 individuals were available) and maintained in aerated and filtered seawater for 24 h up to the start of the experiment.

Odour choice tests were conducted (Wiesemeier et al. 2007) in order to distinguish positive and negative chemotaxis, mediated through the VOCs of *Cocconeis scutellum parva*. They may demonstrate odour preferences resulting from taste-receptor-mediated effects on odour patch residence time. The odour choice assays were performed in Petri dishes (diameter 14 cm, height 2 cm, total volume 307 ml) in a room at 22°C. Each Petri dish was filled with 200 ml of filtered seawater (Sanders™ Protein Skimmer, Eheim™ mechanical-adsorbent filter, and UV steriliser) and placed on a paper sheath printed with 2 experimental arenas (Fig. 1a). Each arena (diameter 14 cm) contained: 2 rectangles at the 2 opposite ends of a diameter, 1 reserved for the sample, the second for the control; a central circle, used for the first deployment of 5 individuals of each species; and 4 vertical lines, delimiting 5 zones. The central zone was ranked zero, and it was the largest, to host most ambiguous individuals and avoid erroneous assignments. The 2 zones near the sample (+) were ranked 1 and 2, respectively. The 2 zones near the control (–) were ranked –1 and –2, respectively (Fig. 1a). The positions of the 2 (+) targets, in the 2 arenas in each sheath, were opposed. Thus, external factors that might influence the movement of animals (e.g. light, magnetism, etc.) were kept random between the replicates, thereby excluding directional effects introduced by the experimental setup.

The agarose gel was prepared before the odour choice assays. We dissolved 1.2 g of low-melt agarose (Sigma A 9045) in 200 ml of filtered and sterilised seawater at 80°C and added 3.3 ml 0.1 M NaOH to adjust the pH (to 8.2–8.4). The agarose solution was poured into a Petri dish and gelled in a refrigerator (5°C) for 8 h prior to cutting the gel into blocks of 0.5 cm². These gels were then used for controls. For experimental treatments, 1, 10 or 100 µl of the VOC bouquet (Table 2) were added to 3 beakers containing still-liquid agarose, after reaching room temperature, 8 h prior to starting the assays. Thus, tests were performed at 3 different concentrations of the diatom VOCs: 0.005, 0.05 and 0.5 µl ml⁻¹ of agarose. The odour bouquet used in the agarose blocks (Table 2) was made from pure compounds and mimicked the molar ratios observed in activated *Cocconeis scutellum parva*. Only those compounds that were available and sufficiently pure (to avoid responses to any contaminating impurity) were included.

After cutting the 0.5 cm² blocks (using a sterilised glass coverslip), glass capillary tubes of 14 cm length were prepared. The odour-containing block of agarose was added to one edge of the capillary glass and a block of control agarose was added to the other edge. This served to fix the position of the blocks in each Petri dish also in the

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Taxon</th>
<th>Feeding habit</th>
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<tr>
<td>1</td>
<td><em>Hippolyte varians</em></td>
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<td>Opportunistic herbivore</td>
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<tr>
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<td>Decapod crustacean</td>
<td>Opportunistic herbivore</td>
</tr>
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<td>Epiphyte grazer</td>
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<td>Gastropod mollusc</td>
<td>Epiphyte grazer</td>
</tr>
<tr>
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<td>Gastropod mollusc</td>
<td>Epiphyte grazer</td>
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<tr>
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</tr>
<tr>
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<td>14</td>
<td><em>Thoralus cranchii</em></td>
<td>Decapod crustacean</td>
<td>Opportunistic detritivore</td>
</tr>
<tr>
<td>15</td>
<td><em>Syllis prolifera</em></td>
<td>Polychaete</td>
<td>Detritivore-herbivore</td>
</tr>
<tr>
<td>16</td>
<td><em>Platynereis sp.</em></td>
<td>Polychaete</td>
<td>Herbivore</td>
</tr>
<tr>
<td>17</td>
<td><em>Symphodus ocellatus</em></td>
<td>Fish</td>
<td>Omnivore</td>
</tr>
</tbody>
</table>
presence of large grazers. Finally, the capillary was positioned in the Petri dish, with the blocks in the appropriate positions according to the targets drawn and indicated as (+) (test sample) and (−) (control), as shown in Fig. 1b. The experiment was started when 5 individuals of each species (Table 1) were added to the central circle of each arena. The position of individuals of each species was recorded after 0, 5, 10, 15 and 20 min, indicating the number of individuals present in each sector (namely the sectors 0, 1, 2, −1 and −2).

**Statistical analysis.** The significance of differences in the distribution of individuals between the control areas (−2, −1) and the VOC areas (+2, +1) in the 6 trials for each species were tested by repeated-measures ANOVA with Prism 4 (Graphpad software). The mean number (with SD) of individuals present in each sector was calculated for each species at each time interval (from 0 to 20 min from the start of the experiment). An index representative of the attraction or repulsion towards the VOC-treated agarose was calculated for each species and each concentration according to the following equation:

\[
\text{Trend} = \frac{NR_2 + NR_1}{2} - \frac{NR_{-2} + NR_{-1}}{2}
\]  

(2)

where \(NR_2\) is the number of individuals in the sector +2, \(NR_1\) is the number of individuals in the sector +1, \(NR_{-2}\) is the number of individuals in the sector −2 and \(NR_{-1}\) is the number of individuals in the sector −1.

The ‘Trend’ scores calculated for each time lapse were then plotted along time, and a linear regression was calculated for each species for each concentration. The slope of each equation represents a synoptic value, indicating the tendency of each species to move towards the positive agarose block (positive value = attractive power), towards the control agarose block (negative value = repellent power) or to remain in the centre, moving around without a clear preference (values close to 0 = no effect).

However, the slope of each linear equation indicates the tendency to move towards a target, not the actual time spent in each sector. In fact, some invertebrates could slowly move towards the positive target, but they may remain most of the time in the negative sectors. Therefore, a second index was calculated as follows:

\[
\text{INT} = \sum (T_1 + T_2 + T_3 + T_4)
\]  

(3)

where \(T_1\), \(T_2\), \(T_3\) and \(T_4\) are the ‘Trend’ values obtained in each of the 4 time lapses (5, 10, 15 and 20 min after the start). The INT index (integral time), therefore, indicates the total time spent by the experimental specimens in different sectors: positive values indicate a higher permanence in the positive sectors (1 or 2); negative values indicate a higher permanence in the negative sectors (−1 or −2); and values close to 0 indicate scarce movements or equivalent movements in the 2 directions.
The 2 scores obtained for each species, at each concentration, were then ordered in an x-y plane, to obtain a comprehensive summary of the results. Ordering of a species in the first sector (positive 'INT', negative slope) indicates a preference for the VOC, but a tendency to move towards the control (see Fig. 3); ordering in the second sector (both positive values) indicates a high preference for the odours and a tendency to move towards the positive agarose block; ordering in the third sector (positive slope, negative INT) indicates that most individuals moved towards the odour sample, but remained most of the time in the negative sectors; and finally, ordering in the fourth sector indicates high repulsive tendency.

**RESULTS**

**Concentration of odour compounds in diatoms**

The diatom yield was 1640 cells mm\(^{-2}\) of the lawn on the bottom of the Petri dishes. Therefore, each Petri dish contained on average \(2.5 \times 10^7\) diatoms. The analysis of all volatile odour compounds irrespective of the presence of given chemical functions was achieved by closed-loop stripping analysis. A typical chromatogram is shown in Fig. 2. Mono-, di- and triunsaturated aldehydes with chain lengths from C\(_5\) to C\(_{10}\) were found in high numbers. Although several saturated aldehydes (heptanal, octanal, nonanal, decanal, undecanal) were also present as prominent peaks in the chromatograms, these compounds were, with the exception of hexanal, regarded as artefacts (Höckelmann & Jüttner 2004). Fatty-acid cleavage products of C\(_5\) and C\(_8\) chain lengths were dominant and contained aldehydes, vinyl alcohols and diones as functional groups. Unexpected was the presence of C\(_9\) (2(E),6(Z)-nonadienal) and C\(_6\) compounds represented by 3(Z)-hexenal, 2(E)-hexenal, hexanal (that may have been contaminated by hexanal as an artefact) and hexan-1. VOCs that were identified by comparison with authentic compounds are shown in Table 3. Monoterpenes and sulphur compounds were not observed in the odour bouquet of *Cocconeis scutellum parva*.

Femtogram amounts per cell were found for pentane-2,3-dione, hexanal, 3(Z)-hexenal, 2(E)-octenal, 3,5-octadien-2-one and 2(E),4(Z),7(Z)-decatrienal. Since the cell volume of *Cocconeis scutellum parva* was determined to be 382 µm\(^3\), the amounts of these components produced per wounded cell were between 9.7 and 80 fg cell\(^{-1}\), corresponding to a concentration of 25 to 209 µmol l\(^{-1}\) when no dilution is taken into
account (Table 3). These concentrations are the maximum that can be expected in a short time reaction period for \textit{C. scutellum parva} that is attacked and disintegrated (see Jüttner 2005). These components are not present in live cells, but they represent the capacity of the cells for odour formation. The unstable \textit{Z}-configuration was largely maintained during the isolation procedure and very little of the unsaturated aldehydes was isomerised to the \textit{E}-configuration, indicating that the analytical method applied was very mild. The isomerisation was 6.8 to 8.8\% for \textit{3(2Z)-hexenal}, 19 to 23\% for \textit{2(E),4(Z)-heptadienal} and 18 to 23\% for \textit{2(E),4(E),7(Z)-decatrienal}.

\begin{table}
\centering
\begin{tabular}{|l|c|c|c|c|c|}
\hline
Compound & \textit{m/z} & \textit{R}_{t} \text{ (min)} & Cellular concentration (ag cell\textsuperscript{-1}) & Theor. conc. (\textmu mol l\textsuperscript{-1}) \\
\hline
1-Penten-3-one & 8.56 & 8.81 & nd & nd & nd \\
Pentanal & 8.74 & 8.98 & nd & nd & nd \\
Pentane-2,3-dione & 43 & 9.16 & 9.37 & 6160 & 8000 \\
2(Z)-Pentenal & 10.81 & 10.97 & nd & nd & nd \\
2(E)-Pentenal & 11.16 & 11.32 & nd & nd & nd \\
1-Hexen-3-one & 33–99 & 11.63 & 11.78 & 76 & 78 \\
Hexanal & 72 & 12.33 & 12.48 & 1560 & 1480 \\
3(Z)-Hexenal & 33–99 & 12.59 & 12.59 & 1780 & 1560 \\
2(E)-Hexanal & 83 & 14.68 & 14.79 & 130 & 150 \\
Hexanol-1 & 69 & 15.79 & 15.94 & 280 & 320 \\
2(E)-Heptenal & 70 & 18.29 & 18.38 & 120 & 84 \\
Octane-2,3-dione & 99 & 19.07 & 19.16 & 400 & 480 \\
1-Octen-3-ol & 57 & 19.22 & 19.32 & 30 & 23 \\
2(E),4(Z)-Heptadienal & 81 & 19.94 & 20.03 & 560 & 740 \\
2(E),4(E)-Heptadienal & 81 & 20.37 & 20.46 & 170 & 170 \\
2(E)-Octenal & 70 & 21.67 & 21.77 & 2600 & 2090 \\
3,5-Octadien-2-one & 33–125 & 22.25 & 22.33 & 820 & 1200 \\
2,5-Octadien-1-ol & 33–127 & 22.57 & 22.63 & 130 & 90 \\
2(E),6(Z)-Nonadienal & 70 & 24.73 & 24.82 & 280 & 350 \\
2(E)-Nonenal & 33–141 & 24.85 & 24.94 & 980 & 760 \\
2(E),4(Z),7(Z)-Decatrienal & 79 & 28.97 & 29.05 & 1280 & 1520 \\
2(E),4(E),7(Z)-Decatrienal & 79 & 29.66 & 29.75 & 390 & 340 \\
\hline
\end{tabular}
\caption{Volatile organic compounds (VOCs) of 	extit{Cocconeis scutellum parva}. Fragment ions or the ranges of fragment ions used for quantitative analyses (m/z), retention times \textit{R}_{t} on a DB 1301 GC column, 30 m length, 0.32 mm inner diameter, 4 min 0\textdegree C, then 5\textdegree C min\textsuperscript{-1} up to 250\textdegree C), and amounts of volatile compounds produced per cell are given for 2 independent determinations using 1 (1P) and 4 Petri dishes (4P). ag = attogram. Theor. conc. = theoretical concentration of individual odour compounds after activation of the lipoxygenase cascade in the cell. nd = not determined}
\end{table}

Odour choice experiments

Choice experiments indicated that some species of gastropod molluscs, such as \textit{Alvania lineata} and \textit{Rissoa variabilis}, are attracted by the \textit{Cocconeis scutellum parva} VOCs even at the lowest concentration (1 µl), while for other species, e.g. \textit{Gammaridea fucicola}, \textit{Hippolyte} spp., \textit{Halacarus} sp., \textit{Gibbula umbilicaris} and \textit{Platynereis} sp., this VOC concentration acts as a repellent (Fig. 3). \textit{Symphodus ocellatus}, a benthic fish, demonstrated a clear attraction to the VOC at the lowest concentration, although its point was ordered in the

![Fig. 3. Ordination into 1 of 4 sectors of species according to the scores reached for ‘Slope’ and ‘INT’ (integral time) (see ‘Statistical analysis’). A, B and C indicate the 3 volatile organic compound (VOC) concentrations tested (0.005, 0.05 and 0.5 µl per ml of agarose gel, respectively). The numbers after these letters indicate the species considered, according to Table 1]
centre of the INT axis, probably due to the continuous movements of fish (faster than any invertebrate) in the experimental arena. These data are in partial agreement with those obtained using an intermediate concentration (10 µl) of VOCs, suggesting that *Symphodus ocellatus*, *Dynamene bifida*, *R. variabilis* and *Syllis prolifera* are the species most attracted by the VOCs of *Cocconeis scutellum parva*. *Gammarella fucicola*, *A. lineata*, *Platynereis* sp., *Gibbula umbilicaris* and *Halacarus* sp. were strongly repelled by the odour compounds at this concentration. The situation differed slightly at the highest concentration (0.5 µl ml⁻¹). Under these conditions, *Halacarus* sp., *R. auriscalpium*, *Gibbula umbilicaris*, *Cestopagus timidus* and *Syllis prolifera* appear to have been attracted by the compound, while *Hippolyte* spp., *Thoralus cranchii*, *R. variabilis* and *Symphodus ocellatus* were repelled by the compound (Fig. 3). When we also consider, however, the slopes (tendency to move towards the VOC sample), *Halacarus* sp., *Cestopagus timidus*, *Pusillina* sp. and *Gibbula umbilicaris* were the most attracted species, while *Hippolyte* spp., *D. bifida*, *A. lineata* and *R. violacea* were repelled by the highest concentration of the odour bouquet (Table 4). In addition, we clearly observed *Symphodus ocellatus* attracted to the VOC as food, at the lower concentrations. In fact, although they continuously moved in the Petri dishes, showing a schooling behaviour, when they approached the (+) agarose they also grazed its surface; this behaviour was never observed in the presence of the control (−) agarose.

**Table 4. Summary of the main effects (attraction or repulsion) observed overall at the 3 different concentrations of volatile organic compounds (VOCs) tested.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Attracted at concentration</th>
<th>Repelled at concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Hippolyte varians</em></td>
<td>A,B,C</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Hippolyte inermis</em></td>
<td>A,B,C</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>Avlania lineata</em></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>4</td>
<td><em>Rissoa variabilis</em></td>
<td>A,B</td>
<td>C</td>
</tr>
<tr>
<td>5</td>
<td><em>R. violacea</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td><em>R. auriscalpium</em></td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>7</td>
<td><em>Pusillina</em> sp.</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td><em>Gibbula umbilicaris</em></td>
<td>C</td>
<td>A,B</td>
</tr>
<tr>
<td>9</td>
<td><em>Cestopagus timidus</em></td>
<td>B,C</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>Gammarella fucicola</em></td>
<td>A,B</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td><em>Bittium reticulatum</em></td>
<td>A,C</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td><em>Dynamene ct. bifida</em></td>
<td>A,B</td>
<td>C</td>
</tr>
<tr>
<td>13</td>
<td><em>Halacarus</em> sp.</td>
<td>C</td>
<td>A,B</td>
</tr>
<tr>
<td>14</td>
<td><em>Thoralus cranchii</em></td>
<td>A,B</td>
<td>C</td>
</tr>
<tr>
<td>15</td>
<td><em>Syllis prolifera</em></td>
<td>A,B,C</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td><em>Platynereis</em> sp.</td>
<td>A,B</td>
<td>C</td>
</tr>
<tr>
<td>17</td>
<td><em>Symphodus ocellatus</em></td>
<td>A,B</td>
<td>C</td>
</tr>
</tbody>
</table>

**DISCUSSION**

A feature of several planktonic and benthic, freshwater and marine diatoms is their ability to produce large amounts of volatile, highly odoriferous, lipoxygenase-mediated unsaturated fatty-acid degradation products. Two major groups of diatoms can be distinguished. One group produces polysaturated cyclic and non-cyclic hydrocarbons in an oxygen-dependent reaction, but it does not produce volatile components with oxygen-containing functional groups (Wendel & Jüttner 1996). The other group of diatoms produces volatiles that are primarily represented by unsaturated aldehydes with C-chain lengths of C7 (heptadienals), C8 (octadienals and octatrienals) and C10 (decadienals and decatrienals). Unsaturated hydrocarbons are missing in this group (Wendel & Jüttner 1996, Wichard et al. 2005). A new chemotype is represented by *Cocconeis scutellum parva*. This diatom produces unsaturated aldehydes with chain lengths in the range between C5 and C10 in high amounts, but no unsaturated hydrocarbons. Many of these compounds are described here for the first time in diatoms. A new group is represented by diketones such as pentane-2,4-dione and octane-2,3-dione. Octane-2,3-dione, which has a pleasant odour, has been observed during mastication of grass (Young et al. 1997) and it can be expected to be a lipoxygenase-mediated compound as well. In addition, we observed the occurrence of monounsaturated aldehydes, among which 2(E)-octenal was released in remarkable amounts. The presence of C6 compounds was unexpected. They are typical components of lipoxygenases of higher plants. These compounds have not yet been described for diatoms or any algal metabolism. Unlike in higher plants (Croft et al. 1993), the unsaturated C6 aldehydes of *Cocconeis scutellum parva* were not reduced to the corresponding alcohols and not esterified with acetate. It is hypothesised that these compounds may mimic the presence of a higher plant (in our case, *Posidonia oceanica*, which also exhibits the higher plant lipoxygenase pathway; data not shown). High lipoxygenase activities have frequently been found for biofilm-forming diatoms (Jüttner & Dürst 1997, Jüttner 2005, Fink et al. 2006), but C6 components seem to be unique for this epiphytic species.

The amount of polysaturated aldehydes per cell found in the present study was in the lower range reported for diatoms (Wichard et al. 2005, Ribalet et al. 2007). However, methodological differences may influence the results and seriously affect the observed amounts of aldehydes. Wichard et al. (2005) activated the lipoxygenase cascades in diatoms by application of the cytotoxic compound O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride. Concomitantly this
reagent efficiently traps the formed aldehydes and protects them from being subject to further reactions and losses caused by their volatility. The molar concentrations of the odour compounds (representing the maximum we may expect in a freshly disintegrated cell suspension of *Cocconeis scutellum parva*) are still high enough to be detected as cues by invertebrates and fish odour receptors. Odour receptors are active in the nanomolar range (Boland et al. 1995) and they allow for an appreciable dilution of the odour bouquet before the detection limit is reached.

The results of the choice experiments confirmed that odour compounds produced by *Cocconeis scutellum parva* are recognised by most invertebrates and trigger different, often unpredicted reactions in individual species. The fish *Symphodus ocellatus* and such invertebrates as *Dynamene bifida*, *Syllis prolifera*, *Rissoa variabilis* and *Cestopagurus timidus* were attracted by the compounds applied at the intermediate concentration. The latter species are herbivores, and the attraction observed, therefore, is according to our expectations. The reaction observed at the highest concentration was not always in accordance with our expectations, since ‘sensitive’ species could be confused in an environment that is over-saturated by the intense odour of these diatoms (Pohnert 2004). In fact, species such as *Symphodus ocellatus* and *Thoralus cranchii* that were attracted by the compound at low and intermediate concentrations, were repelled at the highest concentration. However, *Halacarus* sp., *R. auriscalpium*, *Gibbula umbilicaris*, *Cestopagurus timidus* and *Syllis prolifera* were attracted by the compound at the highest concentration, and they are all hosted in the leaf stratum of *Posidonia oceanica*, and considered typical grazers of the epiphytic layer. Therefore, we can argue that some grazers are particularly accustomed to the odour of chewed diatoms (Fink et al. 2006) and probably attracted by other animals grazing in the same area, following an odour patch. A similar behaviour was observed in the mangrove snail *Terebralia palustris* by Fratini et al. (2001): active feeding of snails on the leaf litter led to the release of odour compounds and subsequently to the attraction of conspecifics.

Interestingly, *Hippolyte inermis* and *H. varians*, both typical hosts of the leaf stratum of *Posidonia oceanica* and grazers of leaf epiphytes (including *Cocconeis* spp. diatoms; Zupo 2001), appear to be repelled by the VOCs. Therefore, repulsion to the odour of the diatoms could appear to contradict previous observations (Zupo 1994). However, the VOCs in this study considered are released in the environment after the wounding of the diatom cytoplasmic membrane (Pohnert 2002, D’Ippolito et al. 2004). Since hippolytid shrimps are subjected to high predation pressure and their behaviour is influenced by the need to avoid predation (Zupo 1994, Bedini et al. 1997, Zupo & Nelson 1997), the diffusion of wound-activated VOCs may indicate the presence of predators chewing parts of *P. oceanica* and its epiphytes.

In contrast, the odour attracts other grazers, e.g. some gastropod molluscs. In fact, gastropods continuously search for exploitable epiphytes and the odour of wounded diatoms indicates abundance of possible food. Molluscs are also very sensitive to the presence of the VOCs and some gastropods positively react to low and intermediate concentrations. Repellence was exhibited by *Gibbula umbilicaris*, but only at the lowest concentrations, while at increasing concentrations this gastropod appeared attracted by the ‘odour’ of diatoms. In this case, the observed behaviour was exactly opposite of that shown by other molluscs (e.g. *Rissoa* spp.). The different localisation of the 2 gastropods along the leaves of *Posidonia oceanica* may explain the opposing reactions observed. In fact, *Rissoa* spp. live in the middle part of the leaf, which is generally covered by diatoms, while *G. umbilicaris* lives in the top part of the leaf, which is heavily epiphysitised by filamentous and encrusting macrophytes (Mazzella et al. 1991).

Therefore the same signal, i.e. the odour of smashed diatoms, may represent an attractant for some invertebrates that need to maximise the search for food, and a repellent for other invertebrates (Fink 2007), for which mimicry and defence from predators represent the most important behavioural constraints (Lamberti et al. 1995). This is not surprising, since the same compound produced by benthic diatoms, e.g. 2(5),4(4)-octadienal, attracts the mollusc *Radix ovata* (Fink et al. 2006), but serves as a repellent for crustaceans at high concentrations (Jüttner 2005).

Some of the compounds produced by diatoms when wounded are defence compounds, such as highly unsaturated fatty acids. For example, 5,8,11,14,17-eicosapentaenoic acid has been shown to be toxic for freshwater herbivores (Jüttner 2001) and we know that this compound is largely present in *Cocconeis scutellum parva* (Nappo et al. 2009). However, since *Hippolyte inermis* normally feeds on these diatoms (Zupo 2000, 2001), it is evident that the same ‘defence’ compounds may have variable effects on individual invertebrate species. Similar substances were also found in freshwater environments (Jüttner & Wurster 1984), and it was demonstrated that diatoms produced these compounds (Wendel & Jüttner 1996, Jüttner & Dürst 1997). In conclusion, as a paradox, VOCs produced by *C. scutellum parva* induce an escape reaction in *H. inermis*, which actively feed on them, but they attract several other grazers, thereby playing the role of foraging kairomones, indicating the presence of abundant food and the possibility to join a grazer’s banquet. The
mosaic of results obtained at various concentrations, in different species, indicates that these wound-activated infochemicals are well-recognised by most invertebrates living in seagrass meadows (Kessler & Baldwin 2001) and they play a variable but important role, producing complex patterns of behavioural reactions and a web of chemical communications (Croll 1983, Watson & Ridal 2004) among diatoms and the invertebrates associated with the leaf stratum of Posidonia oceanica.

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Chapter 6

ECOLOGICAL ROLE OF BENTHIC DIATOMS AS REGULATORS OF INVERTEBRATE PHYSIOLOGY AND BEHAVIOUR

Valerio Zupo* and Chingoileima Maibam

ABSTRACT

Diatoms play several ecological roles in the marine benthos as well as in the plankton. Some diatoms have a deterrent power against grazers as they produce toxic aldehydes and other compounds able to reduce the viability of planktonic copepod embryos. Correspondingly, diatoms of the genus Cocconeis and in particular C. scutellum parva and C. neothumensis, are able to selectively destroy the androgenic gland (AG) and the testis of the shrimp Higpalyne inermis, so determining its early sex reversal, in the field and in the laboratory. These effects are due to a specific apoptogenic activity affecting the shrimp's AG in a very narrow temporal window. Extracts of these diatoms also trigger the quick apoptosis of selected cancer cells. The still unknown active compound might have, therefore, interesting biotechnological applications. In addition, it was demonstrated that among the wound-activated compounds characterizing several benthic diatoms, also a large set of volatile organic compounds (VOC) exists. VOCs produced by Cocconeis spp. after wounding influence the behaviour of several benthic invertebrates, acting as a repellent or attractant according to the life strategy of individual species. In this chapter we review our knowledge about the "regulatory" influences of diatom metabolites on benthic invertebrates, discussing their role as both physiologic modulators and infochemicals.

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INTRODUCTION

The influence of diatoms on the reproductive ecology and the life cycle of planktonic crustaceans has been previously demonstrated [1, 2, 3]. The production of diatom compounds detrimental to the development and survival of grazers has major impacts on plankton secondary production [4]. In addition, studies on the effects of polyunsaturated aldehydes (PUA) on such benthic organisms as tunicates [5], echinoderms and polychaetes [6, 7] indicate that their effect could be as acute in benthic as in planktonic animals. As for the benthos, sub-lethal concentrations (1.32 μM) of decadienal (an aldehyde contained in some planktonic diatoms) induced the formation of abnormal tadpole larvae in the tunicate Ciona intestinalis, with developmental aberrations, abnormal sensory organ pigmentation and reduced elongation of the tail. It prevented the embryonic development prior to reach the gastrula stage [8]. Sea urchins (Paracentrotus lividus) larvae showed malformations at the same concentrations [7]. Abnormal development of Psammechinus miliaris echinopluteus larvae was also observed after sub-lethal (respectively 0.66, 3.29 and 6.58 μM) exposure to decadienal. Larvae of polychaetes are sensitive to aldehydes as well [9], since even a 0.06 μM decadienal solution (much lower concentration in respect to that inducing abnormalities in sea urchins) induced morphological abnormalities during embryogenesis in 9-day old larvae of Nereis virens. This indicates large differences in sensitivity to aldehyde exposure among benthic organisms. In comparison, the concentration of the same aldehyde, able to trigger malformations and tissue degeneration in pelagic copepods, is higher.

In Hippolyte inermis Leach, 1815, a small benthic shrimp living in seagrass meadows of the Mediterranean sea and along the Atlantic coast of Spain [10] individuals were observed experiencing a male stage prior to switching to female [11, 12] due to a protandric sex reversal process [13]. It is well known [14, 15] that its juvenile diet is based on microalgae. Sex differentiation occurs at a size of 5-7 mm [12], while sex reversal is observed in individuals of 10-13 mm, corresponding to the age of 7-12 months. However, not all individuals exhibit sex reversal. In fact, young females of 5-6 mm size are present in natural populations. They are smaller than any male and derive from direct differentiation. Large females deriving from sexual inversion were designated as alpha females, while small females, apparently produced by direct development, were designated as beta females [16]. The period of maximum abundance of beta females in natural populations corresponds to a massive epiphytic production in the leaf stratum of P. oceanica [17, 16]. It was demonstrated that the presence of beta females is due to the ingestion of some diatoms (Cocconeis spp.) that trigger a quick destruction of the androgenic gland (AG) by apoptosis in young males, and the consequent shift to female sex [18]. Therefore, the influence of diatom compounds on the sex reversal of this shrimp plays an interesting and peculiar ecological role and it might lead to biotechnological applications.

In fact, biomolecules having medical applications are widely distributed in marine organisms, and there is a renewed interest in obtaining natural products and better understand the chemistry of medicinal products and foods. Some planktonic diatoms contain compounds able to produce apoptosis of neoplastic cell lines [19]. The anti-cell-growth activity of 3 diatom aldehydes (2-trans-4-cis-7-cis-decatrienal, 2-trans-4-trans-7-cis-decatrienal, and 2-trans-4-trans-decatrienal) was tested on animal models. They all reduced egg-hatching rates in copepods and inhibited cleavage in sea urchin embryos when
concentrations were higher than 0.5 µg ml\(^{-1}\). In Caco2 cell lines derived from a human colon adenocarcinoma, the concentration of aldehydes required to arrest cell growth was 11-17 µg ml\(^{-1}\). However, these toxic apoptogenic compounds were detected in planktonic diatoms, since they synthesize unsaturated aldehydes from fatty acids through various enzymatic pathways, as also reported for marine higher plants, for reducing the grazing activity. The case *Hippolyte-Coccoeneis*, in the benthos, has a different significance, due to the co-evolution and adaptation to informational compounds used to synchronize population dynamics [20] through the apoptosis of the AG and the male gonad. Therefore different compounds [21], even if starting from similar biochemical pathways, play a similar physiological role (apoptosis), have a different ecological significance, and open newer frontiers in the biotechnological application of apoptosis to a broad spectrum of disciplines, from medicine to aquaculture.

However, wound-activated compounds may play additional roles for benthic invertebrates [22]. Several compounds deriving from the lipoxygenase cascade are volatile organic compounds (VOC) acting as important infochemicals in biofilms of benthic primary producers. They can play a steering role of foraging kairomones in marine and freshwater benthic habitats [23], as it has been established for many organismic interactions in terrestrial ecosystems. These compounds may be recognized by various organisms and have the role of "odours", in the sense that they may trigger escape or attraction reactions, according to the life strategy and the needs of different species of invertebrates.

Biological functions of VOCs in aquatic ecosystems are becoming clear [24] and, besides the apoptogenic activity previously introduced, an alternative role of polyunsaturated aldehydes is serving as repellents for crustacean grazers [25]. Aquatic systems are ideally suited for communication by means of chemical cues, because infochemicals can be easily distributed in sufficient concentrations for a response [26]. In particular, VOCs remain spatially localized in highly structured and stable benthic ecosystems like seagrass meadows, in respect to planktonic ecosystems, and they may produce microzones [27]. However, we cannot exclude a role for VOCs as infochemicals also in the planktonic environment, since this aspect has never been investigated. Therefore, the chemical stimuli affecting the behaviour and the chemotaxis of gastropods, decapods, isopods and other invertebrates will be discussed, as an alternative ecological function of wound-activated compounds.

**ALGAE-ASSOCIATED MICROORGANISMS**

Prior to describing the effect of marine drugs on various invertebrates, we should highlight that most diatoms and other producers of bioactive compounds may harbour microorganisms on their surface, including bacteria, cyanobacteria and fungi [28, 29]. The relationships between diatoms and other marine microorganisms that live either permanently or temporarily on their surface are complex and partially unknown, but the latter may be involved in the biosynthesis of various active compounds [30]. Therefore the real identity of bioactive compound producers may be confused. However, in this review we will consider only compounds that are certainly synthesized by selected diatoms, after wounding of their cell walls or mechanical damage [23].
**SEXUAL DIFFERENTIATION IN DECAPOD CRUSTACEANS**

We have considerable proof for the control of sexual differentiation in crustaceans. The development of oocytes in the ovary results from an auto-differentiation process in the absence of male hormone (AGH), whatever the genetic sex [31, 32]. In fact, sexual differentiation in crustaceans is regulated by the androgenic gland (AG), which plays a pivotal role in the regulation of male differentiation and in the inhibition of female differentiation [33, 34, 35]. AG is the unique source of the hormones responsible for the sex differentiation, *i.e.*, the commitment of an embryo to either the female or the male pathway [36].

![Image of Androgenic gland of Hippolyte inermis](image)

*Figure 1. Androgenic gland of Hippolyte inermis observed in a 5 µm section after staining with ematoxilin-eosin. Longitudinal lateral sections of *H. inermis* male. SD, sperm duct; Tes, testes; AG, androgenic gland.*

In male crustaceans the endocrine and gametogenic functions are clearly separated into two distinct organs, the AG and the testis, respectively [37, 33]. Thus, the sex differentiation may be manipulated through the removal of the AG, without damaging the gonad tissues. An AG hormone (AGH) was purified in three isopods [38, 39, 40]. It was demonstrated that the androgenic gland hormone (AGH) of the isopod Armadillidium vulgare is a heterodimeric
glycopeptide [41]. Other investigations [42] revealed an AG-specific gene termed Mr-IAG (i.e., *Macrobrachium rosenbergii* insulin-like AG) gene. Therefore, it is not surprising that the ultrastructure of the AG in different crustaceans resembles that of a vertebrate protein-producing tissue [43]. Additional histological evidences confirm the presence of a proteinaceous androgenic hormone in *M. rosenbergii* [43, 44, 45]. Complete amino acid sequencing showed a structure that seems to be a pro-AGH in the form of a protein containing three peptide chains, and it has been suggested that this complex is the AGH precursor.

The initiation and intensity of spermatogenic activity are regulated by circulating AGH [33]. Spermatogenesis starts only when the AGs are fully developed, in certain decapod species [46, 47]. On the other hand, in the male prawn *M. rosenbergii* [48] and in intersex individuals of the Australian red claw crayfish *Cherax quadricarinatus*, removal of the AG leads to cessation or regression of spermatogenesis, retardation of the testes [49] and to the development of female primary and secondary sex characters [34, 50]. In *M. rosenbergii*, AG removal from immature males resulted in sex reversal with complete female differentiation, while AG implantation into immature females led to the development of a male reproductive system. Sex-reverted *M. rosenbergii* were capable of mating and producing offsprings. Similarly, the administration of apoptogenic compounds present in benthic diatoms induce the quick regression of the small AG in young specimens of the shrimp *Hippolyte inermis* (Figure 1), and the complete female differentiation [21], while individuals fed on diets without diatoms develop as males and revert their sex after several months [51].

![Figure 2](https://example.com/image.png)

**Figure 2.** Life cycle of *Hippolyte inermis* according to [16]. Three subsequent years (Y1, Y2 and Y3) are considered. P₁ and P₉ refer to the male and the female generations which produce the first offspring. F₁ₐ and F₁ₜ refer to the generations observed in the first year. F₂ₐ and F₂ₙ refer to the generations born in the year 2.
THE SEX REVERSAL IN *Hippolyte inermis* LEACH 1815

As a matter of fact, sex reversal is common among decapod crustaceans [52]. Within the multivariate and complex sexual strategy adopted by decapods [53], which includes gonochorism, sequential and simultaneous hermaphroditism and even parthenogenesis [54], *Hippolyte inermis* is a puzzling case. Two main periods of recruitment, in spring and fall, were detected in the life cycle of *H. inermis* [16]. Individuals born in spring grow quickly and develop as both females or males, while individuals born in fall grow slowly and develop as males, reverting sex in the next spring (Figure 2). It was demonstrated that the guts of individuals undergoing early sex reversal (beta females) contained a high abundance of diatoms of the genus *Cocconeis* [55]. In addition, it was demonstrated in the laboratory that young specimens fed on diets containing *Cocconeis* diatoms developed mainly as females, while most individuals fed on diets not containing diatoms developed as males [18]. Therefore, it was concluded that the ingestion of some diatoms induced the production of beta females, as observed in spring generations in the field (Figure 3).

![Figure 3. Role of diatoms in the sex reversal of *Hippolyte inermis* ([55], modified). In the upper part, the gut contents obtained from individuals collected in the field in the critical months for recruitment show dramatic differences in the abundance of diatoms fed. In the lower part, the main results of a laboratory experiment are reported, to show that individuals fed on diatoms developed mainly as females (left) while individuals fed on basic foods without diatoms developed mainly as males (right).](image)

This species is characterized by a singular feature [11]: female gonads are not produced through the development of an ovotestis (as in other sex-reverting invertebrates; [52]). They
are built up from stem cells conserved in the adult male. In fact, it was demonstrated that the effect of compounds contained in some benthic diatoms (Cocconeis) is directed towards the AG, destroying it during its development [56]. According to the hormonal regulation described in the previous section, common to all crustaceans, the lack or the ablation of the AG induces the development of females. It was recently demonstrated [18, 56] that this effect is produced by apoptosis (programmed cell death). Ingested diatoms contain compounds able to selectively destroy cell populations, inducing their suicide [21]. The action of the considered apoptogenic compound is species-specific, dose-dependent and extremely selective for the AG. It triggers the quick death of cells naturally programmed to die about 12 months after hatching. Various diatoms are able to influence this process, but their level of efficiency varies (Figure 4), being some species of Cocconeis the most effective. Interestingly, when planktonic diatoms (Skeletonema costatum) toxic for copepods were administered to H. inermis, they did not show any activity on the sex reversal process, nor triggered a significantly higher mortality ([21]; Figure 4) in respect to controls. For this reason, C. scutellum parva and C. neothumensis were considered to be the species containing the highest concentrations of the active compound [56]. The identification of the peculiar pro-apoptotic factor contained in these diatoms might be crucial for several medical purposes. For the same reason, investigations are presently carried out, aimed at identifying the apoptogenic compound, elucidating its chemical structure and understanding its molecular mechanism of action.

**Treatments**

Figure 4. Variable efficacy of several species of diatoms, both benthic (Navicula sp., Diploneis sp., Cocconeis scutellum parva and C. neothumensis) and planktonic (Skeletonema costatum), in the production of beta females ([21], modified). Asterisks indicate significant differences in respect to controls (CTRL).
KNOWN MECHANISMS OF ACTION OF DIATOM WOUND-ACTIVATED COMPOUNDS

Eukaryotic algae have evolved complex systems by which the formation of deterrent compounds is primed by the activity of grazers [57]. Two induced reaction chains of microalgae are well known:

a) The dimethylsulfiniopropionate (DMSP) pathway (the compound is transformed into dimethylsulfide and acrylate, catalyzed by the enzyme DMSP lyase).

b) The lipoxygenase cascade, liberating a greater number of compounds, several of which may have biological activity. Some lipids are hydrolyzed upon disintegration of the cell walls, and they are transformed into unsaturated fatty acids, readily oxidized to hyperoxy fatty acids and, in a subsequent cleavage reaction, to unsaturated aldehydes and ω-oxo-fatty acids [58, 59].

In this review we will mainly take into account the pathway (b), because it is the one commonly observed in diatoms (Figure 5). It is more complex than the DMSP reaction. Diatom cell damage (e.g., after wounding) activates lipase enzymes that liberate polyunsaturated fatty acids (PUFAs) from cell membranes. Among these, some PUFAs are precursors of PUA through the lipoxygenase activity, and they may be potentially harmful to developing embryos and larvae. In fact, they are readily oxidized and cleaved to form PUA and other metabolites (collectively termed oxylinps). Various planktonic micro-organisms may produce similar compounds and effects. Previous investigations [60] revealed abnormal development in embryos and larvae of the sea urchin Paracentrotus lividus incubated with the fatty acid octadecapentaenoic acid (C18:5 n-3), derived from a dinoflagellate (Gymnodinium sp.).

![Diagram](image)

**Figure 5.** Biosynthetic pathway for oxylinps starting from fatty acids in marine diatoms. R1 represents the methyl terminal part and R2 represents the carboxylic end of C16 or C20 fatty acid precursors. R1 and R2 may differ in length and degree of unsaturation ([59], modified).
Type and quantity of wound-activated compounds differ among species and strains, due to a variety of metabolic pathways; as variable are their effects on individual planktonic or benthic grazers. Oxylinins, and aldehydes, in particular, exhibit biological activities ranging from the total block of gametogenesis, abnormal gamete functionality and fertilization, to errors in the embryonic mitosis, low larval fitness and competence [8]. Arrest of cell cleavage has been reported both in Paracentrotus lividus and Sphaerechinus granularis eggs treated with decadienal [19, 61]. At higher concentrations decadienal induces apoptosis in P. lividus embryos. In addition, decadienal inhibits tubulin polymerization, DNA synthesis and cyclin B/Cdk1 kinase activity, leading to the arrest of the cell cycle in S. granularis early embryos [62]. Interestingly, only aldehydes bearing an α,β- or α,β,γ,δ-unsaturated structural element show a biological activity, whereas saturated and unsaturated aldehydes, which lack such a Michael acceptor system, exhibit no activity at all [63].

A possible explanation for the difference in activity between various groups of PUFAs may be their tendency to form hydroperoxides, due to a higher unsaturation. The biological activity of PUFAs is due to modifications of the cell membrane structure, alterations of the membrane protein functions and of passive and active ionic transport, also in relation to calcium conductance [64]. Altering ion balance and calcium conductance leads to cell death and, in fact, some of the compounds having effects on sea urchin development are omega 3 fatty acids.

Hydroperoxides formed from EPA have been shown to be very active in blocking copepod early development compared to aldehydes and other oxylinins [59]. It was demonstrated that 5,8,11,14,17- eicosapentaenoic acid (EPA), one of the main precursors of diatom PUAs, as well as 4,7,10,13,16,19-docosahexaenoic acid (DHA), 6,9,12,15-octadecatetraenoic acid (stearidonic acid), 6,9,12-octadecatrienoic acid (γ-linolenic acid) and 9,12-octadecadienoic acid (linoleic acid) blocked sea urchin cell cleavage. The toxicity increases according to chain length from C7 to C10 PUAs, since arrest occurred at 27.27 μM with heptadienal, 16.13 μM with octadienal, 11.47 μM with octatrienal and 5.26 μM with decadienal. Sub-lethal concentrations of these compounds induced programmed cell death in embryos and larvae [8]. Interestingly, EPA is a central compound in the biosynthesis pathways of several diatoms [57] and it is also present in high proportion in Coccconeis scutellum, the diatom inducing the sex reversal of the shrimp Hippolyte inermis [65]. Therefore, the same compound, or its derivatives, is active in different environments (plankton vs. benthos) and in different organisms, from copepods to decapods and sea urchins. However differences in the activity between EPA and its methyl ester were also detected. A drastic reduction in the activity of the methyl ester may be due to physico-chemical properties of the two molecules, leading to distinct interactions with the cell membrane [66].

In conclusion, if we compare the effects on sea urchin development, it is clear that aldehydes have a more pronounced and specific effect on embryonic and larval development, with at least one order of magnitude higher activity in blocking development, as compared to PUFAs. These relationships may lead to biotechnological applications. Dietary supplementation with EPA significantly increased the levels of this fatty acid in the mucosa of human colon. This evidence was associated with reduced proliferation and increased mucosal apoptosis, indicating the potential efficacy of EPA supplementation in the chemoprevention of colorectal cancer [66]. Other compounds that are part of the biosynthesis
pathway of EPA, even more specific than this fatty acid, could find interesting biomedical applications.

**APOPTOGENIC EFFECT OF WOUND-ACTIVATED COMPOUNDS**

The homeostasis in several physiological states is maintained by means of the programmed cell death (apoptosis). This refers, for example, to the embryogenesis or the achievement of hormonal signalling, although the role of apoptosis in marine ecology is still scarcely known. Apoptosis has been shown to play two major roles during the development: removing damaged cells during embryogenesis and sculpturing tissues during morphogenesis and metamorphosis [67]. However, apoptosis is also induced by various stress factors, including toxicants, pollutants and heat shock. Only recently, it was demonstrated [1] that some plankton diatoms are able to induce apoptosis in the embryos of marine copepods, reducing their viability: egg production and hatching success in the copepod *Calanus helgolandicus* are negatively influenced by the ingestion of diatoms of the genus *Thalassiosira*. The apoptosis induced by planktonic diatoms is triggered by α, β-unsaturated aldehyde and seems to be caspase-independent [68].

Figure 6. *Vas deferens* (d) and testis (T) of an *Hippolyte inermis* postlarva (PL6) after feeding on *Cocconeis scutellum*. Whole individual stained by the TUNEL technique, observed at the complanar microscope (Leica Z16-APO) under UV light, to detect apoptosis by fluorescence (light areas).
As previously highlighted, the role of apoptosis in the interaction *Hippolyte-Cocconeis* has a different ecological significance, due to co-evolutionary processes (both species are well adapted to the seagrass environment). In fact, the toxic effect of diatoms is translated into a spring signal for the development of beta females [20]. However, also in this case the biological effect (early occurrence of the sex reversal in *Hippolyte inermis* postlarvae) is due to an apoptotic activity, able to selectively destroy the androgenic gland (AG) in a very narrow temporal window, *i.e.*, from the second to the eight day of postlarval development ([56]; Figure 6). Investigations are still in progress to identify and elucidate the structure of the pro-apoptotic compound producing these effects. Noteworthy, various marine natural products induce growth arrest and apoptosis in sea urchin embryos [8] as well as in human neoplastic cells *in vitro* and *in vivo* [69, 70] and they demonstrated strong activity against cancer, with lower toxicity if compared with traditional chemotherapeutic agents. Some examples are aplidin, a marine compound isolated from *Aplidium albicans*, and ecteinascidin-743 derived from *Ecteinascidia turbinata* [71, 72 73]. Additional roles of apoptosis in ecological fields are still to be investigated, but the examples here reported indicate that this mechanism could play a major role in regulating the relationships among plant and animal species within marine benthic and planktonic communities.

**ROLE OF WOUND-ACTIVATED COMPOUNDS AS INFOCHEMICALS**

Besides the above-mentioned direct effects on the physiology of diatom consumers, secondary metabolites may represent information means, triggering reactions of escape or attraction in various grazers [22]. Volatile organic compounds released from benthic algae are suitable for dispersion over distance, and they may indicate the presence of algal food. A complex web of trophic, physiological, spatial and chemical relationships is produced by the stable coexistence of associated plant [74] and animal [75] populations. Compounds responsible for the biological effects have been demonstrated to be volatile organic compounds (VOC) not present in intact cells, but released by a lipooxygenase cascade upon cell wounding [76, 57].

Secondary metabolites produced by the lipooxygenase pathway may also act as repellents against grazers [23]. Therefore, it is evident that diatoms producing volatile compounds have a range of possible consequences. Co-evolutionary processes may modulate their effects, as is the case of VOCs that have been shown to play a role for locating suitable habitats for aquatic insects and nematodes [77]. To investigate these effects in the benthos, *Cocconeis scutellum parva* was isolated, cultivated and the volatile compounds released upon cell disintegration were identified by mass spectrometric methods [22]. The presence of unsaturated aldehydes with chain lengths from C5 to C10 was demonstrated. Unexpectedly, C6 compounds were found, represented by 3(Z)-hexenal, 2(E)-hexenal, hexanal and hexanol-1, that resemble the typical bouquet observed in higher plants. These may be responsible for a strategy of chemical mimicry adopted by diatoms living on *Posidonia oceanica* leaves. Compounds not described before for diatoms were, among others, pentane-2,3-dione and octane-2,3-dione. Food choice experiments performed on 17 animal species associated with *P. oceanica* meadows indicated that these grazers recognize the presence of the diatom odour compounds, exhibiting complex patterns of reactions according to their life strategies [22].
Interestingly *Hippolyte inermis* and *Hippolyte varians*, both typical hosts of the leaf stratum of *P. oceanica* and grazers of leaf epiphytes (including *Coccoeis* diatoms; [55]), were repelled by the diatom VOCs. Therefore, repulsion to the odour of the diatoms could appear to contradict previous observations [16, 55]. However, the VOCs here considered are released in the environment after the wounding of the diatom cytoplasmic membrane [78, 79]. Since hippolytid shrimps are subjected to a high predation pressure and their behaviour is influenced by the need to avoid predators [16, 80, 81], the diffusion of wound-activated VOCs may indicate the dangerous presence of predators chewing parts of *P. oceanica* and its epiphytes. In contrast, the odour attracts other grazers, e.g., some gastropod molluscs. In fact, gastropods continuously search for exploitable epiphytes and the odour of wounded diatoms indicates abundance of possible food.

Our results confirmed that odour compounds produced by *C. scutellum parva* are recognized by most invertebrates and trigger different, often unpredicted reactions in individual species [22]. The same signal may represent an attractant for grazers that need to maximise the search for food, and a repellent for other species [82], for which mimicry and defence from predators are the most important behavioural constraints (Figure 7). The mosaic of results obtained at various concentrations, in different species, indicates that diatom wound-activated infochemicals are well recognized by most invertebrates living in seagrass meadows [83] and they play a variable but important role, producing complex patterns of reactions and a web of chemical communications [84, 85] among the invertebrates associated with the leaf stratum of *P. oceanica*.

![Figure 7. Effects produced (attraction vs. repulsion) by the exposition of various benthic invertebrates to the odour bouquet (VOCs) of *Coccoeis scutellum parva*. The A/R index was calculated as an average of the slopes obtained at 3 different concentrations [22]. Bars in the right sector indicate that the species is attracted to the odour and their size indicates the level of preference. Bars in the left sector indicate that the species is repelled by the odour and their size indicates the level of repulsion.](image-url)
CONCLUSION

Some compounds produced by diatoms upon wounding are defence compounds, such as highly unsaturated fatty acids. For example, 5,8,11,14,17-eicosapentaenolic acid (EPA) has been shown to be toxic for freshwater herbivores [57] and we know that this compound is abundant in Cocconeis scutellum parva [65] as in most other diatoms and various microalgae. However, since the shrimp Hippolyte inermis normally feeds on these diatoms [18, 55], it is evident that the same “defence” compounds may have variable physiologic effects on individual invertebrate species: the apoptogenic compound (not yet identified) abundant in few species of Cocconeis is important to keep a sufficient number of primary females in the natural populations of H. inermis [16, 18]. Therefore, in this case, a deterrent compound has been transformed into a spring signal for the production of primary females [21]. In other cases, as observed in planktonic copepods, similar compounds deriving from the lipoxygenase cascade biosynthetic pathway, play an effective physiologic role by reducing the reproduction success of their grazers. The data reported here suggest that VOCs are not only important information-transmitting cues in terrestrial ecosystems [86], but also in benthic marine and freshwater habitats. In this case, it was demonstrated that they could modulate the behaviour of individual species of invertebrates and trigger reactions of attraction or escape, so contributing to the structure shaping, at a small spatial scale, of benthic associations.

Therefore, wound-activated compounds synthesized through the lipoxygenase cascade are among the most interesting compounds produced by diatoms, exhibiting physiologic effects on invertebrates and possible biotechnological applications. In most diatoms this activation produces a range of PUFAs, PUAs and hydroperoxides. The toxicity of PUAs increases shifting from C7 to C10 molecules while, as for aldehydes, only those bearing an α,β- or α,β,γ,δ-unsaturated structural element show a clear biological activity. A thorough investigation of these relationships, as well as a deeper comprehension of the mechanisms leading to a very specific apoptogenic activity, as the one observed in the case of Hippolyte inermis and Cocconeis spp., will be crucial for understanding the effect of diatom compounds on individual benthic invertebrates, as well as for exploiting related biotechnological applications.

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Chemoreception of the Seagrass *Posidonia Oceanica* by Benthic Invertebrates is Altered by Seawater Acidification

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Abstract Several plants and invertebrates interact and communicate by means of volatile organic compounds (VOCs). These compounds may play the role of infochemicals, being able to convey complex information to selected species, thus mediating inter- or intra-specific communications. Volatile organic compounds derived from the wounding of marine diatoms, for example, carry information for several benthic and planktonic invertebrates. Although the ecological importance of VOCs has been demonstrated both in terrestrial plants and in marine microalgae, their role as infochemicals has not been demonstrated in seagrasses. In addition, benthic communities, even the most complex and resilient, as those associated to seagrass meadows, are affected by ocean acidification at various levels. Therefore, the acidification of oceans could produce interference in the way seagrass-associated invertebrates recognize and choose their specific environments. We simulated the wounding of *Posidonia oceanica* leaves collected at two sites (a control site at normal pH, and a naturally acidified site) off the Island of Ischia (Gulf of Naples, Italy). We extracted the VOCs and tested a set of 13 species of associated invertebrates for their specific chemotactic responses in order to determine if: a) seagrasses produce VOCs playing the role of infochemicals, and b) their effects can be altered by seawater pH. Our results indicate that several invertebrates recognize the odor of wounded *P. oceanica* leaves, especially those strictly associated to the leaf stratum of the seagrass. Their chemotactic reactions may be modulated by the seawater pH, thus impairing the chemical communications in seagrass-associated communities in acidified conditions. In fact, 54% of the tested species exhibited a changed behavioral response in acidified waters (pH 7.7). Furthermore, the differences observed in the abundance of invertebrates, in natural vs. acidified field conditions, are in agreement with these behavioral changes. Therefore, leaf-produced infochemicals may influence the structure of *P. oceanica* epifaunal communities, and their effects can be regulated by seawater acidification.

Keywords Acidification · *Posidonia oceanica* · Wound-activated · VOC · Invertebrate · Seagrass · Odor · Infochemical

Introduction

The acidification of oceans, due to increasing levels of CO₂ in the atmosphere and surface oceans (Brewer 2013), may interfere with the lives of various organisms and communities, due both to chemical influences imposing physiological adaptations and to changed relationships among organisms, affecting their communications and coexistence (Fabricius et al. 2014; Kroeker et al. 2011; Wyatt et al. 2014). Most seagrasses have been demonstrated to be able to thrive at high levels of CO₂ (Apostolaki et al. 2014; Garrard et al. 2014), and to survive in areas constantly characterized by low pH (Gartner et al. 2013). However, their epiphytic communities are dramatically influenced by seawater at low pH, since various calcareous algae may be selected against, even by slight pH changes (Donnarumma et al. 2014; Martin et al. 2008). The invertebrate community of the leaf stratum is associated strictly to the epiphytic community (Lebreton et al. 2009), both for shelter
and food availability (Mazzella et al. 1992). Therefore, a slight lowering of pH, still insufficient to influence the growth of a seagrass, may have dramatic effects on the animal assemblages normally present, leading to a simplified trophic structure (Kroeker et al. 2011). In this case, the species diversity and the abundance of invertebrates may remain almost unchanged (Garrard et al. 2014).

We also know that associated invertebrates recognize the seagrass meadows, where they find food, shelter, and protection from predators, due to visual and chemical stimuli (Zupo and Nelson 1999). It is further known that seagrass tissue composition (e.g., the abundance of phenolic compounds) changes according to the ambient pH, and that lower amounts of deterrent compounds are produced by P. oceanica living in an acidified environment (Arnold et al. 2012; Garrard and Beaumont 2014) as well as by other seagrasses (Campbell and Fourqueuran 2013). Therefore, the tissues of seagrasses living in an acidified environment may play different functional and structural roles (Jernakoff and Nielsen 1998) compared to plants growing in normal conditions (Mazzella et al. 1992), although this aspect has not been investigated. This effect is added to the direct influence of changes in the associated epiphytic cover that reduces the shelter for animals and the complexity of food webs. These changes influence as well the nutritional value and the C/N ratios of epiphytes (Ricevuto et al. 2015).

The ability of algae to produce volatile organic compounds (VOCs) that trigger specific reactions in some invertebrates has been demonstrated recently (Maibam et al. 2014). Individual species of invertebrates are attracted by the odor of specific microalgae because they may represent feasible food, or be deterred by the odor of other algae because their wounding may indicate the presence of predators (Jüttner et al. 2010).

Volatile organic compounds produced by wounded tissues are different from and have a different role, in respect to constitutive metabolites present in the tissues of various organisms (Thoms and Schupp 2008). In fact, constitutive compounds often are confined in the tissues, not diffusing into the environment, when the plant cells are intact. In the case of constitutive emissions, the diffusion is not linked to specific events generated by plant-animal relationships (Grote et al. 2013; Monson et al. 2012; Niinemets et al. 2013), as is the case of wound-activated compounds. In contrast, the wounding of plants represents a dynamic event in the frame of plant-animal relationships, and its recognition may be crucial for some invertebrates to identify food sources or the presence of possible predators (Dicke and Sabelis 1988). Thus, several wound-activated VOCs play the role of infochemicals (Maibam et al. 2015), and they may trigger specific reactions in selected invertebrates (Fink 2007). In addition, the recognition of specific bouquets of odors depends on the ecological relationships of invertebrates with their environment (Maibam et al. 2014), and the perceptive abilities of invertebrates are inversely correlated to the toxicity of wound-activated compounds (WACs).

Volatile organic compounds are quickly delivered to target organisms, even at a long distance from the source of the “odor” (Kaasik et al. 2011; Lewis et al. 2012), and they often play the role of infochemicals. Chemoreception in terrestrial and aquatic complex ecosystems requires the detection of small differences in mixture composition, as opposed to the recognition of a few specialized compounds (Horner et al. 2006). Consequently, invertebrates exclusively or frequently associated to a given seagrass meadow should be able to recognize its “odor”, since they have evolved specific chemoreceptive abilities (Jüttner et al. 2010) towards a bouquet of various VOCs produced upon wounding of the plant tissues. In fact, the wounding activity may indicate the presence of consumers or predators (Pohnert et al. 2007), requiring an active reaction by the target organisms. However, we still need investigations to establish whether invertebrates are able to recognize (Pohnert et al. 2007) the odor of a wounded seagrass, and if specific reactions may be induced by higher plant infochemicals. As well, it is still unknown whether seagrass tissue modifications influenced by the acidification of oceans may trigger changes in the reactions of associated invertebrates.

It is important to stress that the behaviors triggered by chemical cues are not only due to the invertebrate’s search for food (Fink 2007), since only a few species directly graze on Posidonia leaves (Mazzella et al. 1992). Indeed, the infochemicals produced by the wounding of leaves can indicate the occurrence of larger organisms (e.g., fishes), and may represent critical signals for the presence of predators (Dolecal and Long 2014).

To understand these fundamental aspects of their complex ecology, we collected Posidonia oceanica leaves at two different sites, one at normal pH (8.1) and the second at low pH (7.7), close to the ocean acidification scenario and conditions forecasted for the end of the present century (Caldeira and Wickett 2005; IPCC 2007). Subsequently, we exposed a set of 13 benthic invertebrates to the VOCs produced, after wounding, by the two types of Posidonia leaves, under normal pH conditions (8.1) and in acidified water (7.7), to understand if they recognized them by interpreting their chemokinetic reactions (Horner et al. 2008). In this way, we expected to detect any variation in respect to “normal” behavior (Weissburg and Zimmer-Faust 1991) due to either i) the different composition of P. oceanica tissue grown in normal pH (8.1) and acidified (7.7) environments, and ii) any chemical modification of the VOCs due to the pH of the medium (8.1 vs. 7.7) or a modification of the invertebrate’s receptors (Wyatt et al. 2014).

The research aimed at checking whether: 1) invertebrates recognize the VOCs produced by wounded Posidonia leaves,
2) the intensity of attraction/repulsion exhibited is according to the degree of association of each species to the leaves of *P. oceanica*, and 3) the responses of invertebrates may explain their abundances in *P. oceanica* either in “normal” or in acidified waters.

### Methods and Material

#### Study Sites

*Posidonia oceanica* leaves were collected at Castello Aragonese, located in the island of Ischia (Gulf of Naples, Tyrrenhenian Sea, Italy). It is represented by a volcanic islet located off the northeast coast of the island (40°43.853′N, 13°57.698′E) and is characterized by the presence of volcanic CO₂ emissions (Tedesco 1996). Due to the gas emissions, the pH of the seawater drops to exceptionally low values, from ambient levels (ca. 8.1) in the control station, that is far from the venting areas, down to 6.4 in the areas of intense venting (Kroeker et al. 2011). Thus, the site shows a permanent pH gradient, from high venting areas to absence of venting, approx. 200 m long, and occurring both off the north and south sides of the islet. It represents a “natural laboratory” simulating the pH conditions forecasted for the future of oceans (Hall-Spencer et al. 2008). We collected *Posidonia* leaves in control areas (pH 8.1), and in the acidified area characterized by a pH close to 7.7 (Garrard et al. 2014).

Invertebrate specimens were collected mainly at Lacco Ameno (Gambi et al. 1992), located on the north-west coast of Ischia (40°45.432′N, 13°53.135′E), approx. 6 km apart from Castello vents. It hosts a well studied *P. oceanica* meadow that extends continuously from 1 to 32 m (Buia et al. 1992; Zupo et al. 2006). The pH of the seawater is stable around 8.1–8.2 (Garrard et al. 2014; Ricevuto et al. 2015), in accordance with the average values for the Mediterranean sea.

#### Collection of Posidonia oceanica

Collections of *P. oceanica* leaves were conducted by SCUBA divers at Castello Aragonese, in two different areas, i.e., in the acidified meadow (pH about 7.7), and in the meadow at normal pH (8.1). Five shoots for each location were selected randomly, collected over a surface area of about 20 m², and immediately transferred to the laboratory. Shoots collected in the acidified area are morphologically different from those living at normal pH, due to a strong grazing activity by herbivorous fish (e.g., *Sarpa salpa*). Therefore, all leaves collected in the acidified area are short cut, and they lack apical tips (Donnarumma et al. 2014).

For our experiments, only the central parts of leaves were considered, in order to avoid comparisons of different portions of the shoots due to the above-mentioned morphological differences. In particular, the lowest 10 cm of intermediate and adult leaves were discarded; the next 20 cm were used for VOC extractions; the remaining tips, when present, were discarded.

Leaves from each shoot were separated, cleaned from epiphytes by means of a steel blade, and then gently cleaned with soft paper to remove any trace of microalgae still present on their surface. Subsequently, the middle sections, as above specified, were cut into small pieces, mixed, and weighed (fresh weight). Three small samples of leaves were wrapped in aluminum foil and dried in an oven (65 °C) until constant weight (dry weight). The fresh weight/dry weight ratio was calculated, to be used for evaluation of the actual biomass tested in our bioassays. The collected leaf portions were frozen immediately (~20 °C) and used for the extraction of VOCs, a few hours prior to the choice tests.

#### Collection of Invertebrates

Collections of invertebrates were performed both at Lacco Ameno, at normal pH conditions, and at Castello Argonese, in the sector characterized by normal pH. Our collections were not quantitative (see Garrard et al. 2014, for a quantitative estimation of invertebrate associations present at Castello Aragonese and Gambi et al. 1992 for invertebrates at Lacco Ameno) since they were used only to provide living specimens for choice experiments. A circular plankton net (1 m frame diam; mesh size 100 μm) was gently trawled horizontally by a research boat over the *Posidonia oceanica* meadow, and invertebrates were collected in a glass jar attached to the end of the net. First sorting of the collected animals was performed on board, and specimens of various taxa were pooled into plastic bags containing clean seawater, and immediately transferred to the laboratory. Additional invertebrates were collected by SCUBA divers, by towing a rectangular framed net over the leaf canopy (Buia et al. 2004).

All invertebrate specimens collected were identified by specialists, *in vivo*, under a stereomicroscope, moved into aerated vessels, and kept in a thermostatic chamber, in the presence of small pieces of *Posidonia* leaves (for shelter and food) up to the day before the experiment. They were starved for 24 h prior to being tested. A species was considered for choice tests when at least 30 individuals were collected and available. Thirteen species were selected, belonging to the main meso faunal taxa present in the seagrass environment, i.e., polychaetes, gastropod mollusks, isopods, amphipods, and decapod crustaceans.

Specimens assayed at low pH conditions were adapted slowly to acidified water (pH 7.7), starting the night before the experiment. After the experiments in acidified water, each specimen was kept at pH 7.7, in glass vessels, prior to being slowly adapted to normal pH (8.1). All specimens still alive were returned to the sea after the completion of the experimental procedures.

#### Extraction of VOCs

Volatile organic compounds (VOCs) were extracted twice from frozen leaves (2 × 4 g DW) of *Posidonia oceanica* after grinding them in a mortar under...
liquid nitrogen. The same weights of leaves were used for extraction from the normal pH site and the acidified site. Thus, the VOCs incorporated into the agarose blocks were proportional to the original concentration of infochemicals in the wounded leaves. Volatile organic compounds were concentrated by closed-loop stripping (Jüttner 1988) performed at 22 °C for 45 min. For this purpose, 8 g of ground and sonicated leaves were suspended into 40 ml of filtered (Millipore 0.22 μm) seawater and transferred to a 100 ml round bottom flask. After addition of 10 g NaCl, the VOCs were extracted and absorbed onto a Tenax TA cartridge (Fink et al. 2006a, b). The cartridge was removed and eluted with 6 ml diethyl ether. The ether was gradually evaporated using nitrogen gas (N₂ and absorbed onto a Tenax TA cartridge (Fink et al. 2006a, b). The cartridge was removed and eluted with 6 ml diethyl ether. The ether was gradually evaporated using nitrogen gas (N₂, and absorbed onto a Tenax TA cartridge (Fink et al. 2006a, b). The cartridge was removed and eluted with 6 ml diethyl ether. The ether was gradually evaporated using nitrogen gas (N₂, grade 5.0), and the residue was re-dissolved in 300 μl of pure ethanol. Controls were prepared according to the same procedure, but stripping was performed on filtered and sterilized seawater without the addition of leaves. All VOC samples and controls were stored at −80 °C until the choice experiments were conducted.

Preparation of Gels Bioassays were conducted as reported below, in static chambers (Jüttner et al. 2010), after the inclusion of VOCs into small blocks of jellified agarose. To this end, we prepared agarose gels added with VOCs and controls. To prepare a 0.06 % agarose gel, 1.2 g of agarose (Sigma A-9045) were dissolved in 200 ml of filtered and sterilized seawater, heated (80 °C), and stirred until completely transparent. The pH of the solution was adjusted to a value close to 8.2 by adding 3.3 ml of 0.1 M NaOH. Controls were prepared by incorporating 250, 25, and 2.5 μl of the above described control extract into liquid (but close to room temperature) agarose, just before gelling, to obtain three concentrations of control gels. The agarose solution then was poured into a Petri dish and allowed to gel in a refrigerator at 5 °C, 1 h prior to the start of assays.

To prepare VOC agarose blocks, 250, 25, and 2.5 μl of the ethanolic VOC extract were incorporated, respectively, into the still liquid (but close to room temperature) agarose, just before gelling. In this way, we obtained three different concentrations, namely, “low”, “medium”, and “high”. The low concentration simulates the VOCs released by 5 mm² of Posidonia leaf wounded by a grazer. The medium concentration corresponds to 50 mm² of Posidonia leaf wounded by a large grazer. The high concentration corresponds to 500 mm² of Posidonia leaf wounded by a herbivorous fish or a similar grazer. Finally, the agarose gel disks were cut (using clean glass coverslips) into small blocks, each measuring 0.5 cm³ and used for the choice tests on invertebrates.

Choice Tests on Invertebrates Thirteen invertebrate species were selected from our samples and identified. The set is representative of different feeding habits and various levels of associations to Posidonia oceanica leaves (Table 1). Therefore, we expected that sensitive organisms would orient, according to their preferences, along the VOC gradient (Chase 1982).

The assays were conducted in 14 cm (diam) Petri dishes set over circular experimental arenas printed on paper sheets, according to the protocols suggested by Jüttner et al. (2010). Each arena consisted of five sectors, (−2, −1, 0, 1, 2) indicating the rate of repulsion or attraction according to the invertebrate movements (Fig. 1). In fact, these annotations refer to the distance from the positive target, being sector +2, the one containing the agarose added with VOCs and the −2, the one containing the control agarose; “0” is the central sector, intermediate between the positive target and the negative control. For the experiments in acidified conditions, Millipore (0.45 μm) filtered seawater was added with CO₂ using a Ferplast CO₂ Energy® reactor, which allows for acidification of seawater by avoiding any bubbling. The water was checked for its pH (7.7) prior to being used for filling the experimental arenas.

Five individuals of each species were released at the center (marked as a circle) of each arena, and they were allowed to perceive the odor of the Posidonia leaves, diffusing from the “+2” target. The number of individuals present in each sector of the arena was recorded at four time intervals (5, 10, 15, and 20 min) from the start of each test. Precautions were taken to minimize any external factor possibly influencing the movement of animals during the experiment, such as light, temperature, magnetism, etc. In particular, experiments were conducted at 18 °C under a well-lit and diffused light, and each of the replicated two arenas were positioned in such a way that the positive targets opposed each other.

Six replicates were obtained for each invertebrate species for each treatment (Table 2). Four treatments were considered: i) VOCs from Posidonia collected at ambient pH tested in Petri dishes containing seawater at normal pH (8.1); ii) VOCs from Posidonia collected at pH 7.7 tested in Petri dishes containing seawater at ambient pH (8.1); iii) VOCs from Posidonia collected at ambient pH tested in Petri dishes containing acidified seawater (pH 7.7); iv) VOCs from Posidonia collected at pH 7.7 tested in Petri dishes containing acidified seawater (pH 7.7). For each treatment, three concentrations were tested (low, medium, and high), corresponding to 5, 50, and 500 mm² of P. oceanica leaves, respectively. In total, 6 replicates of 12 treatments were tested on 13 invertebrate species (Table 2). When some invertebrates had to be tested again under different treatments, they were allowed to rest overnight in a thermostat chamber (18 °C 12:12 h L/D) prior to start the new experiment. The assemblages of 5 individuals used for each replicate were randomly re-assigned every time.

Calibration of Experimental Vessels The choice experiments were performed based on the assumption that VOCs added to an agarose gel may diffuse producing a continuous
gradient, as suggested by Steinke et al. (2002). Nevertheless, the behavioral response of animals within each test may or may not justify this assumption. In order to confirm the presence of a VOC gradient, we performed a calibration experiment prior to the start of the choice tests, by using a well-known VOC that is easily quantifiable by means of spectrophotometric analyses.

For this purpose, agarose blocks were loaded with the volatile compound 2-trans-4-trans-decadienal (Sigma-Aldrich), an aldehyde produced by some planktonic diatoms upon wounding (Wichard et al. 2005). In particular, 150 μl of decadienal, previously dissolved in 4 ml of methanol, were added to 200 ml of freshly prepared agarose, in order to obtain a final concentration of 240 μg/ml. The agarose gels were refrigerated and cut into 0.5 cm³ blocks, as reported for the experimental VOCs. The blocks then were positioned in the (+) targets of the experimental arena, while negative blocks (agarose gels) were positioned in the (−) targets. Four sets of 3 replicated arenas were prepared contemporaneously. The vessels were positioned on a squared matrix, containing the indication of 30 sampling points. Every 5 min, a set of 3 replicates was sampled by means of an automatic pipette, and 1 ml of the medium was collected, from each sampling point on the grid. These collections were repeated at 5, 10, 15, and 20 min. The collected solution then was spectrophotometrically analyzed (Hewlett Packard 8453 spectrophotometer) at a wavelength of 282 nm, and the concentration of decadienal was calculated.

Table 1 Thirteen species of macroinvertebrates tested for their responses to volatile compounds produced by three benthic diatoms

<table>
<thead>
<tr>
<th>Nr</th>
<th>Taxon</th>
<th>Species (References)</th>
<th>Trophic habits</th>
<th>Assoc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Polychaete</td>
<td>Platynereis dumerilii</td>
<td>herbivore</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Polychaete</td>
<td>Keferssteinia c. c.</td>
<td>omnivore/carnivore</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Isopod</td>
<td>Dynamene bijda</td>
<td>herbivore</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Amphipod</td>
<td>Caprella acanthifera</td>
<td>omnivore</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Amphipod</td>
<td>Gammarrella fucicola</td>
<td>herbivore</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Decapod</td>
<td>Hippolyte inermis</td>
<td>herbivore/omnivore</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>Decapod</td>
<td>Cestopagurus timidus</td>
<td>omnivore/carnivore</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>Decapod</td>
<td>Calcinus tubularis</td>
<td>omnivore/carnivore</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>Gastropod</td>
<td>Rissoa italensis</td>
<td>herbivore</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>Gastropod</td>
<td>Rissoa variabilis</td>
<td>herbivore</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>Gastropod</td>
<td>Rissoa violacea</td>
<td>herbivore</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>Gastropod</td>
<td>Bittium latreelli</td>
<td>detritus feeder</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>Gastropod</td>
<td>Gibbula umbilicaris</td>
<td>herbivore</td>
<td>3</td>
</tr>
</tbody>
</table>

Nr.: reference to the invertebrates as used also for cluster analysis. The level of association to Posidonia oceanica meadows is categorized in the last column (assoc.): 1, present; 2, typical; 3, generally abundant; 4, almost exclusive.

Fig. 1 Experimental arenas used for choice experiments. Animals were deployed in the central circle at the start of the experiment. The VOC-added block of agarose was fixed into the “+” square. The control agarose was fixed into the “−” square. Test invertebrates were counted every 5 min into the sectors, according to their ranking, from −2 to +2.

Table 2 Experimental plan

<table>
<thead>
<tr>
<th>pH of P. oceanica collection site</th>
<th>Treatments (performed at 3 concentrations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.7</td>
<td>pH 8.1 (A - C)</td>
</tr>
<tr>
<td>8.1</td>
<td>pH 7.7 (C - A)</td>
</tr>
</tbody>
</table>

Posidonia oceanica leaves were collected at two stations at Castello Aragonese, characterized by different pH (first column), and choice experiments (treatments) were conducted, for both collections, in experimental vessels filled with seawater at two different pH (last two columns). Each treatment was repeated at three concentrations of VOCs (low, medium and high).

A acidified, C control.
(Pippen and Nonaka 1958) according to its molar epsilon 31, 000 m$^{-1}$ cm$^{-1}$ of optical density (OD) referred to a lambda max of 274 nm in methanol.

All concentrations obtained were recorded into a matrix indicating the position of samples, and the average of three replicated samples was calculated. These values were computed using the Krigeing technique (Matheron 1969, 1970) that allows a spatial representation of the concentration, considered as a stationary phenomenon. In this way, we obtained a map of the concentrations measured in each point of the experimental arenas, described by isolines, suitable to confirm the exactness of our hypotheses and to track the diffusion gradient from the agarose gels in the Petri dishes during the experiment.

**Statistical Analyses** To compare the reactions of invertebrates towards the odor of *Posidonia* leaves collected at two different sites (normal and low pH), under two experimental conditions (normal pH and acidified water), we calculated and plotted the average Preference Index (P.I., as proposed by Jüttn er et al. 2010) exhibited by each tested invertebrate and the standard error. The latter was chosen according to previous investigations (James et al. 2008) because the individual variability characterizing ethological responses is well known, and we aimed at showing the average level of preference of each species, not the obvious scattering of results due to the natural random invertebrate movements. The significance of the results provided by each choice test was evaluated by means of the Z-test on proportions, by comparing the proportion of individuals present in the “+” sectors at the end of the experiment to the proportion present in the “−” sectors. The test indicates whether the observed distribution is significantly different from a normal distribution of individuals (Sprint hall 2011).

In addition, a matrix “treatments vs. arena sectors” was filled and submitted to cluster analysis (by means of STATISTICA 10, StatSoft) to allow grouping of the species tested according to their patterns of reactions in the various experimental trials.

**Results**

Calibration of the experimental vessels confirmed the production of a VOC gradient initially concentrated into the “++2” sector (Fig. 2a), slowly diffusing towards the “−−2” sector (Fig. 2b, c) according to the time of sampling. After 20 min (Fig. 2d), the gradient was still present, but the differences between the two extremes of the experimental vessel were reduced, as compared to the start of the assay.

The choice tests yielded complex patterns of reactions, variable in each invertebrate species according to the concentration, the type of *Posidonia* tissues, and the pH of the medium.

*Posidonia oceanica* leaves sampled at normal pH prompted some evident and significant reactions, indicating that several invertebrates recognize their odor (Fig. 3). In particular, *Platynereis dumerilii*, *Kefersteinia cirrata*, *Dynamene bifida*, and *Gibbula umbilicaris* exhibited a significant reaction of attraction towards the odor of *P. oceanica* at low concentration, while *Rissoa italiensis* and other gastropods showed slight repulsion (Fig. 3a). At the medium concentration, the leaves of *P. oceanica* produced more repulsion, and, limiting our examination to significant results, we observed (Fig. 3b) that *Gammarella fucicola*, *Cestopagurus timidus*, *Calcinus tubularis*, and *Bittium latreilli* were repelled by *P. oceanica* VOCs, while *Rissoa violacea* was attracted.

The choice tests conducted at high concentration yielded the largest number of significant reactions, but some invertebrates exhibited contrasting reactions in respect to those reported for the low concentration. Besides polychaetes, isopods and amphipods, which elicited a positive chemotactic reaction at all concentrations, mollusks and decapods showed a repulsive reaction (significant in the case of *G. umbilicaris*, *B. latreilli*, *R. italiensis*, *C. tubularis*, *C. timidus*, and *H. inermis*) at different concentrations (Fig. 3b, c). In the case of *B. latreilli*, the repulsive effects of *P. oceanica* VOCs were proportional to the concentration: apparent low repulsion (P.I. −0.33, but not significant) at low concentration, higher repulsive reaction (P.I. −0.66, significant) at the medium concentration, and the highest repulsive reaction (P.I. −1.2, significant as well), at high concentration.

Similar relationships were observed for species attracted by *P. oceanica* VOCs, although the results obtained at medium and high concentration are generally in better agreement. For example, in the case of *Kefersteinia cirrata*, attraction was demonstrated by a preference index of 1.16, 0.62, 0.81, respectively, at low, medium, and high concentration, exhibiting a decrease at medium and high concentrations in respect to the low concentration. The same trends were shown by other polychaetes, amphipods, isopods, and decapods. In the case of *H. inermis*, for example, low concentration of VOCs triggered slight attraction (Fig. 3a), medium concentration triggered a lesser attraction (Fig. 3b), and high concentration triggered an escape reaction (Fig. 3c).

Consistently, a decreasing trend of attraction was observed moving from low towards high concentrations, when *P. oceanica* leaves collected at normal pH were tested in a medium at pH 7.7, although some invertebrates reversed their reactions (Fig. 4). In fact, most invertebrates exhibited a significant positive reaction at high concentration (Fig. 4c): the responses of polychaetes, amphipods, isopods, decapods, and most mollusks (with the exception of *Rissoa violacea*) were in agreement with the results obtained for the low concentration tested at normal pH (Fig. 3a). In contrast, low and medium concentrations of VOCs triggered less obvious reactions (Fig. 4a, b).
Aquatic different patterns of chemotactic reactions were prompted by VOCs extracted from P. oceanica leaves collected in acidified conditions (pH 7.7) and tested at the same pH (Fig. 5a). Most species exhibited a low-level response at low concentration, and only Gammarella fucicola and Hippolyte inermis were significantly attracted by the VOCs, while Cestopagurus timidus was significantly repelled. A larger number of invertebrates exhibited a significant chemotactic response at “medium” concentration of VOCs (Fig. 5b). In fact, the two amphipods both exhibited a low but significant repulsion, along with the mollusk Rissoa variabilis. Hippolyte inermis and Calcinus tubularis showed a clear positive chemotaxis. The high concentration disoriented most invertebrates: only three significant responses were exhibited (Fig. 5c) by Cestopagurus timidus, Calcinus tubularis (repulsion), and Rissoa italiensis (attraction).

The same tests, performed in normal seawater, yielded a complex pattern of results. Three species (Gammarella fucicola, Cestopagurus timidus, and Gibbula umbilicaris) showed significant attraction, while Rissoa violacea showed rejection at low concentration (Fig. 6a). Consistently, a larger number of species exhibited significant reactions at medium concentration (Fig. 6b). The two polychaetes showed attraction along with Gammarella fucicola, Calcinus tubularis, and Rissoa italiensis. Cestopagurus timidus, in contrast, was repelled by these VOCs tested at normal pH. Only four species reacted at high VOC concentrations (Fig. 6c). Hippolyte inermis, Cestopagurus timidus, and Rissoa variabilis were attracted by acidified Posidonia leaves, while Calcinus tubularis was repelled in these conditions. The chemotactic responses observed are, overall, in accordance with the distribution of the considered species at ambient pH and in the acidified area (Table 3).

The cluster analysis performed on the matrix of preference indices shows two main groups of invertebrates, separated based upon their specific reactions. The first group contains P. dumerilii, C. tubularis, G. fucicola, K. cirrata, and D. bifida (Fig. 7). These species are all characterized by a low level of...
association to *P. oceanica* (Table 1). The second group contains all the other species, mostly characterized by a higher level of association with *P. oceanica* (Table 1).

**Discussion**

**Invertebrate Reactions** The analysis of invertebrate behavior in response to chemical stimuli is complex, due to individual variability and to stochastic factors that influence the animal chemotaxis (Fink 2007; Zhou and Rebach 1999). Several superimposed factors affect the behavior of invertebrates and contribute to the results reported above (Briffa et al. 2012). In addition, chemical cues may be influenced by local ecological conditions that affect the quality and quantity of VOCs produced by plants, as well as the perceptive abilities of individual species of invertebrates (Wyatt et al. 2014).

Nevertheless, some clear and significant trends were observed and, given the experimental procedures followed, we are able to discriminate the effects attributable to changes in

![Fig. 4](image-url) Results of the choice experiments performed on volatile organic compounds (VOCs) extracted from *Posidonia oceanica* leaves collected at normal pH (pH 8.1) and tested in acidified seawater (pH 7.7). Bars indicate attraction (positive bars) or repulsion (negative bars) according to the preference index proposed by Jüttner et al. (2010). a. Tests performed at low VOC concentration. b. Tests performed at medium VOC concentration. c. Tests performed at high VOC concentration. * indicates significant results at *P*<0.05; ** indicates significant results at *P*<0.01. When not indicated, results obtained are not significant (*P*>0.05). Standard errors (*N*=6) are indicated by error bars.

![Fig. 5](image-url) Results of the choice experiments performed on volatile organic compounds (VOCs) extracted from *Posidonia oceanica* leaves collected in the acidified site (at pH 7.7) and tested in acidified seawater (pH 7.7). Bars indicate attraction (positive bars) or repulsion (negative bars) according to the preference index proposed by Jüttner et al. (2010). a. Tests performed at low VOC concentration. b. Tests performed at medium VOC concentration. c. Tests performed at high VOC concentration. * indicates significant results at *P*<0.05; ** indicates significant results at *P*<0.01. When not indicated, results obtained are not significant (*P*>0.05). Standard errors (*N*=6) are indicated by error bars.
the VOC composition of Posidonia leaves grown in acidified conditions as compared to leaf tissues living in normal conditions. As well, we can detect any change in the animal’s ability to perceive the same odors at two different pH levels. From these data, we forecast how this issue will affect the structure of future animal communities associated to P. oceanica due to ocean acidification. Several invertebrates showed specific reactions according to the intensity of the infochemical signal (three concentrations) and the four experimental conditions (Posidonia growing at pH 8.1 or 7.7, tested at pH 8.1 and 7.7).

The Polychaete Platynereis dumerilii It is often present but not strictly associated to P. oceanica. It was able to identify the leaf odor, consistently, at all concentrations, at ambient pH. A lower pH produced a reduction in the sensitivity to detect the odor of normal P. oceanica leaves. In fact, VOCs of leaves grown at normal pH were not recognized at low concentration when tested at pH 7.7, while a clear attraction (as observed at normal pH) was recorded at medium and high VOC concentrations. In contrast, P. oceanica growing in acidified conditions produces a different set of secondary metabolites.

Table 3 Comparison of the main results of the choice experiments with the actual abundance of each invertebrate in the acidified site

<table>
<thead>
<tr>
<th>Species</th>
<th>Normal (pH 8.1)</th>
<th>Acidified (pH 7.7)</th>
<th>Actual abundance in the acidified meadow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platyneis dumerilii</td>
<td>Attraction</td>
<td>Repulsion</td>
<td>Low</td>
</tr>
<tr>
<td>Kefersteinia cirrata</td>
<td>Attraction</td>
<td>Reduced attraction</td>
<td>Low</td>
</tr>
<tr>
<td>Dynamene bifida</td>
<td>Attraction</td>
<td>Reduced attraction</td>
<td>Low</td>
</tr>
<tr>
<td>Caprella acanthifera</td>
<td>n.a.</td>
<td>n.a.</td>
<td>–</td>
</tr>
<tr>
<td>Gammarella fucicola</td>
<td>–</td>
<td>Attraction</td>
<td>High</td>
</tr>
<tr>
<td>Hippolyte inermis</td>
<td>–</td>
<td>Attraction</td>
<td>–</td>
</tr>
<tr>
<td>Cestopagurus timidus</td>
<td>n.a.</td>
<td>n.a.</td>
<td>–</td>
</tr>
<tr>
<td>Calcinus tubularis</td>
<td>n.a.</td>
<td>n.a.</td>
<td>–</td>
</tr>
<tr>
<td>Rissoa italensis</td>
<td>n.a.</td>
<td>n.a.</td>
<td>–</td>
</tr>
<tr>
<td>Rissoa variabilis</td>
<td>–</td>
<td>Repulsion</td>
<td>Low</td>
</tr>
<tr>
<td>Rissoa violacea</td>
<td>n.a.</td>
<td>n.a.</td>
<td>–</td>
</tr>
<tr>
<td>Bittium laterelli</td>
<td>Repulsion</td>
<td>Attraction</td>
<td>Low (*)</td>
</tr>
<tr>
<td>Gibbula umbilicaris</td>
<td>Attraction</td>
<td>Attraction</td>
<td>Equal</td>
</tr>
</tbody>
</table>

Both types of Posidonia tissues (collected at normal pH and in acidified conditions) are considered in the first and second columns, respectively, to show the general trends observed. An evaluation of the abundance of each invertebrate in the acidified site, in comparison to the “normal” one, is given in the third column.

*: This is a species linked to the detritus more than the leaf stratum of P. oceanica.

Fig. 6 Results of the choice experiments performed on volatile organic compounds (VOCs) extracted from Posidonia oceanica leaves collected in the acidified site (at pH 7.7) and tested in normal seawater (pH 8.1). Bars indicate attraction (positive bars) or repulsion (negative bars) according to the preference index proposed by Jüttner et al. (2010). a. Tests performed at low VOC concentration. b. Tests performed at medium VOC concentration. c. Tests performed at high VOC concentration. * indicates significant results at P<0.05; ** indicates significant results at P<0.01. When not indicated, results obtained are not significant (P>0.05). Standard errors (N=6) are indicated by error bars.
(Arnold et al. 2012), and its VOCs were scarcely or not recognized by *P. dumerilii*, on the whole. This trend corresponds with the species distribution in the field (Table 3). In fact, the polychaete is present (even if not abundant) in meadows under normal conditions, as in Lacco Ameno (Gambi et al. 1992), but it is rare, in *Posidonia* meadows under acidified conditions at Castello (Garrard et al. 2014; Gambi unpublished data). However, this species is able to grow in acidified water and, in fact, is present in the rocky reef communities off Castello acidified site (Kroeker et al. 2011), as well as on artificial settlement collectors deployed in acidic conditions (Ricevuto et al. 2014). Based on the reactions to the VOCs above reported, we hypothesize that the lower abundance, as observed in acidified conditions, is due to scarce attraction prompted by the VOCs of acidified *Posidonia* tissues. Nevertheless, the reduction in abundance may be influenced as well by the reduced abundance of epiphytic food observed under acidic conditions (Hall-Spencer et al. 2008).

**The Polychaete *Kefersteinia cirrata*** A similar trend is shown by another polychaete tested. In this case, an evident attraction was observed at low concentration in normal conditions, while a higher dose of odor was necessary to trigger a positive chemotaxis in acidified conditions. Contrasting and generally non-significant results were obtained by testing *P. oceanica* tissues grown in acidified waters. On the whole, this suggests that the attractive power of leaf-derived odors is lost under acidified conditions. This species is a carnivore predator (Gambi et al. 1992), and the attractive power of *Posidonia* leaves at low concentration may indicate shelter and adequate habitat.

**The Isopod *Dynamene bifida*** A strong attraction for leaves from the non-acidified site was observed in the case of *D. bifida*, under both normal and acidified conditions, but only at low concentration. *Dynamene bifida* preferentially feeds on algal epiphytes and possibly on scraped tissues of the outermost seagrass leaf layer (Lorenti, pers. obs.). In this case, a moderate release of VOCs may be sensed as a signal of food availability. In contrast, higher VOC concentrations disorient the animals, possibly indicating the presence of predators. The tests performed with leaves from the acidified site showed contrasting results, suggesting a lower sensitivity of the animals to volatile compounds released by their tissues or, alternatively, a lower amount of VOCs produced by *P. oceanica* growing at lower pH. Overall, the reactions triggered by normal *P. oceanica* leaves were of attraction, while those triggered by leaves grown in acidified water indicate that these tissues were not recognized by *D. bifida*. This species is poorly represented in samples from the *P. oceanica* canopy, while it is abundant on algal substrates growing in the acidified sector of Castello (Lorenti, unpublished data) and on artificial collectors deployed in the same zone (Cigliano et al. 2010; Ricevuto et al. 2012). This suggests that, although generally tolerant towards acidification, this species responds differently to the compounds released by the dominant plants at normal pH and in acidified conditions.

**The Amphipod *Gammarella fucicola*** The behavioral patterns exhibited were quite different: this is an herbivore scarcely associated to the leaf stratum of *P. oceanica* (Scipione 2013) and it showed only little response towards VOCs produced by wounded leaves of “normal” *Posidonia*, except for some attraction exhibited in acidified conditions at
high concentrations. Therefore, in this case, the animals were not attracted by the smell of wounded leaves, but seawater acidification modified the pattern. This scheme corresponds to the higher presence of G. fucicola in the Posidonia meadow at Castello, in the acidified site (pH 7.7), while it is less abundant in the meadow growing at normal (8.1) pH (Gambi et al. 1992; Scipione, unpublished data). Therefore, also in this case, the results of choice experiments (slight attraction triggered by “normal” Posidonia, but higher attraction by “acidified” Posidonia) fit the data about the spatial distribution of the species.

The Decapod Hippolyte inermis This shrimp, closely associated to P. oceanica meadows, was not attracted by leaf VOCs under normal pH conditions or at lower concentrations, while it was repelled at high concentration of VOCs. This apparent paradox is in agreement with the results by Jüttner et al. (2010). In fact, it was observed that Cocconeis scutellum parva, a diatom “mimicking” the VOCs of Posidonia and living in the leaf stratum, triggered an escape reaction. This is related to the life strategy of the shrimp, ruled by the avoidance of predators. Low levels of P. oceanica wounded leaves may represent the normal environment for the shrimp, and they do not trigger any definite chemotaxis. However, larger abundances of wounded leaves may represent a danger signal (e.g., a large fish crushing leaves, or other possible predators) and this may prompt escape behavior. As explained in the methods, the three concentrations adopted here simulate, respectively, the grazing by a small invertebrate, a large invertebrate, or some fish. Similarly, a large abundance of wounded diatoms trigger escape behavior (Jüttner et al. 2010), although these microalgae are an important food for the shrimp.

The attraction to leaves became more evident when the tests were performed in acidified seawater, while the escape reaction at high concentration was not significant. On the whole, acidified leaves produced VOCs that were attractive for H. inermis, thus modifying the chemotactic patterns observed under normal conditions. This might hamper the shrimps’ ability to detect and avoid the presence of predators. Similar flawed reactions due to the water acidification, possibly hampering the ability of animals to detect their predators, have been observed for the clownfish Amphiprion percula (Dixson et al. 2010).

The Gastropod Rissoa variabilis This is a small-sized mollusk associated to leaves of P. oceanica and it showed no significant reactions under normal conditions at the lower concentrations, but was clearly repelled by the VOCs produced by P. oceanica under acidified conditions. In this case, the medium pH was responsible for the reaction, because both types of leaves (collected at normal and low pH) appeared attractive at higher concentrations, when tested at pH 8.1, but they repelled the mollusk when tested at pH 7.7. These observations are in agreement with the distribution of the species in the acidified meadow off Castello, in comparison to the meadow growing at normal pH (Garrard et al. 2014). This distribution mainly refers to adult individuals, corresponding to the size classes we tested in the choice experiments.

The Gastropod Bittium latreilli An opposite trend was shown by B. latreilli. It generally was repelled by the leaves of “normal” Posidonia, but was slightly attracted by leaves grown under acidified conditions. This trend appears in contrast with the natural distribution of the species, since a lower abundance of specimens was observed in the acidified site (Garrard et al. 2014), in respect to P. oceanica growing under normal conditions. However, we must consider that B. latreilli differs from the other species here considered. It is typical of a detritus environment, not dependent upon the leaf stratum (Russo et al. 2002). Therefore, the abundances reported by Garrard et al. (2014) must be attributed to the attraction towards VOCs produced by leaf detritus, not by intact leaves. The leaves growing in acidified conditions actually lost several deterrent compounds (Buia unpublished data), and, thus, their bouquet may be more similar to detritus in respect to intact leaves living under normal conditions (Arnold et al. 2012). Our data appear consistent with the natural distribution of the species, although further tests are needed, taking into account the VOCs produced by leaf detritus.

The Gastropod Gibbula umbilicaris It exhibited a clear attraction at low concentration, both towards normal tissues and for leaves grown in acidified waters. Its reactions were inverted at higher doses, probably due to VOC saturation of the experimental arena, that produces disorientation in sensitive organisms (Jüttner et al. 2010). An alternative explanation, taking into account the three ecological scenarios we simulated, is that higher doses indicate the presence of large predators. In this case, the pH does not seem to influence the reactions of the mollusk. In fact, the natural distributions of this species in the acidified site and the “normal pH” are quite similar (Garrard et al. 2014). Thus, the distribution of the species, strictly associated to the leaf stratum, also is in agreement with its reaction to the odor of P. oceanica leaves.

Other Species In the case of the anomurid decapods Cestopagurus timidus and Calcinus tubularis, as well as for the mollusks Rissoa italiensis and R. violacea, contrasting results were observed among the various treatments. Given the scarce statistical significance of most tests (a clear attraction was observed only in acidified conditions, at some higher concentration), it would be hard to draw accurate conclusions: possibly these species, not exclusive of the seagrass environment, have little sensitivity to the VOCs produced by Posidonia leaves.
General Trends Overall, our tests indicate that most invertebrates are able to recognize both the VOCs produced by *P. oceanica* grown under normal conditions and those produced by leaves grown under acidified waters. Most of them responded differently to the bouquets of odors produced by normal and acidified *Posidonia* leaves, and exhibited clear reactions, according to the dose of VOCs to which they were exposed. Most sensitive invertebrates exhibited a clear reaction at low VOC concentrations, while acidification in several cases reduced the ability of animals to perceive these volatile compounds. In these cases, a reaction (of attraction or escape) was observed, at low pH, only at higher concentrations.

Notably, VOCs produced by “normal” *Posidonia* leaves were recognized by 5 species (38% of the examined invertebrates) mostly associated to the leaf stratum, while VOCs produced by leaves growing in acidified environment were recognized by 8 species (62% of those examined). The inofchemical function of the VOCs produced by *P. oceanica* leaves was not lost in acidified conditions (Table 3). However, in some cases, a different strength was shown or the reaction was inverted. In particular, in two cases, grazers gave a weaker response when tested at pH 7.7, while in three cases the bio-assays in acidified conditions showed a stronger response in respect to the test performed at normal pH. The response of two invertebrates was totally inverted in acidified conditions. On the whole, seven species (54% of the tested invertebrates) exhibited different responses in acidified conditions, in respect to normal behavior at pH 8.1.

The behaviors triggered by chemical cues were not only due to the invertebrate search for food; in several cases they can be explained possibly by the life strategies of each species, and represent critical signals for the presence of predators (Doleacal and Long 2014) or by the availability of shelter.

Hence, we here demonstrated that leaf-produced inofchemicals have the potential to influence the structure of *P. oceanica* epifaunal communities, as is known for other environments (Vos et al. 2006). The attraction/repulsion reactions observed are in agreement with their abundance in meadows under normal and acidified conditions. In contrast, leaf-produced inofchemicals have scarce influence on species not strictly associated to the leaf stratum. Seawater acidification has been shown to alter invertebrate behavior in several cases and, in some instances, these modifications have hampered the abilities of invertebrates to detect and avoid their predators, or their skills to perceive the right food (Doleacal and Long 2014). The results of choice tests are in agreement with the actual distribution trends and the abundance patterns of each species found in *P. oceanica* grown in “normal” and in acidified waters.

The data suggest that the increasing levels of CO₂ forecasted for the next decades could dramatically modify (Steinke et al. 2002) the composition and the spatial distribution of animal communities associated to this fundamental seagrass for the Mediterranean coastal system.

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Relevance of wound-activated compounds produced by diatoms as toxins and infochemicals for benthic invertebrates

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Relevance of wound-activated compounds produced by diatoms as toxins and infochemicals for benthic invertebrates

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Abstract Plants evolve the production of wound-activated compounds (WACs) to reduce grazing pressure. In addition, several plant-produced WACs are recognized by various invertebrates, playing the role of infochemicals. Due to co-evolutionary processes, some invertebrates recognize plant infochemicals and use them to identify possible prey, detect the presence of predators or identify algae containing various classes of toxic metabolites. Different metabolites present in the same algae can play the role of toxins, infochemicals or both simultaneously. We investigated the infochemical activity of compounds extracted from three diatoms epiphytes of the seagrass Posidonia oceanica, by conducting choice experiments on invertebrates living in the same community or in close proximity. Furthermore, the specific toxicity of the extracts obtained from the same algae was tested on sea urchin embryos using a standard bioassay procedure, to detect the presence of toxins. The comparison of the two effects demonstrated that invertebrates are subjected to diatom wound-activated toxicants when these algae are not associated with their own habitat, but they are able to recognize volatile infochemicals derived from diatoms associated with their habitats. The specific toxicity of WACs was shown to be inversely correlated to the perceptive ability of invertebrates towards volatile compounds liberated by the same algae. Hence, when the recognition of specific algae by a given invertebrate species evolves, their detrimental effects on the receiving organism may be lost.

Introduction

Diatoms are an important component of marine food webs (Steele 1974), and they represent one of the main food sources in the marine planktonic environment, as well as for several benthic grazers (Mazzella and Russo 1989). They exhibit strong mechanical defences (Hamm et al. 2003) that partially protect them from small grazers (Sunda and Shertzer 2012), but larger herbivores are able to crush the silica frustules and exploit this trophic resource. Therefore, mechanical defences are often insufficient, and diatom survival and bloom formation are only possible because of high intrinsic growth rates and efficient nutrient uptake (Round et al. 1990; Stevenson et al. 1991). Several autotrophs are also known for their ability to produce deterrent compounds (Leflaive and Ten-Hage 2009a). Diatoms, for example, produce toxic oxylipins, including polyunsaturated aldehydes (PUAs) and oxo-acids (Miralto et al. 1999; Wichard et al. 2005a; Fontana et al. 2007a). Oxylipins are released after cell damage (e.g. wounding) through an enzyme cascade, during which polyunsaturated fatty acids (PUFAs) naturally present within cell membranes are rapidly oxidized to form the toxic end products (Pohnert 2000; d’Ippolito et al. 2003). Besides oxylipins and PUAs, diatoms produce other deterrent compounds upon cell wounding (Pohnert 2000) and this production is linked to seasonal variations, strains, light irradiance and other environmental factors (Taylor et al. 2009). All these compounds have
strong effects on grazers, as revealed by studies showing reduced copepod hatching success and/or egg production on diatom-dominated diets. Miralto et al. (1999) demonstrated that inhibitory PUAs blocked embryogenesis in copepods and sea urchins, although other authors proposed alternative roles for aldehydes (Dutz et al. 2008). Several mechanisms have been suggested to explain these negative effects (Poulet et al. 2007).

Various co-evolutionary processes may have modified these relationships and some invertebrates are able to detoxify plant metabolites (Lauritano et al. 2011, 2012; Taylor et al. 2012), as diatoms are an important component of their diet (Zupo et al. 2007). *Hippolyte inermis*, for example, a decapod living in *Posidonia oceanica* (L.) Delile meadows, takes advantage of an apoptogenic compound (Zupo et al. 2014) produced by diatoms of the genus *Cocconeis* to change sex in some seasons (Zupo 2000): this mechanism awards stability to its natural populations, because the apoptosis of the androgenic gland, followed by the destruction of the testis, triggers the production of early females, bursting a fall reproduction season in the Mediterranean (Zupo and Messina 2006).

Several animals evolved the ability to detect and recognize volatile compounds generated by wounded diatoms, using them to analyse the environment and make important decisions (Fink 2007). They live in environments suffused with infochemicals, and the information network can be influenced by both predators and their prey (Vos et al. 2006). Therefore, secondary metabolites produced by microalgae upon wounding may be fundamental for shaping food webs and structure the spatial distribution of invertebrates, at a small scale (Vos et al. 2006). Previous findings indicate that a relationship may exist between the presence of toxic compounds in selected diatoms and the production of volatile organic compounds (VOCs), interpreted by animals as warning or attraction signals. In particular, some invertebrates evolved the ability to recognize selected infochemicals (e.g. volatile compounds) to detect the presence of activated defences (Legrand et al. 2003; Pohnert et al. 2007). For example, Jüttner et al. (2010) demonstrated that the “odours” (volatile organic compounds) of wounded *Cocconeis* spp. are recognized by several invertebrates that individually show attraction or escape reactions, according to their specific ecological needs. These studies indicated that diatoms produce several secondary metabolites, especially when they are wounded (activated defences), which include nutrients and toxins (Dicke and Sabelis 1988). However, these compounds are sometimes detoxified by grazers and subsequently may become infochemicals (Leflaive and Ten-Hage 2009b). For this reason, chemicals produced by marine microalgae received increased attention in the last decade (Ianora et al. 2011), for their role in shaping interactions and community structures (Jüttner 1999; Fink 2007; Flynn and Irigoien 2009).

A close relationship could exist in marine diatoms between the presence of activated defences (deterrent and toxic wound-activated compounds, WCs) and volatile infochemicals (VOCs). Volatile compounds, in fact, are not always toxic (Fink 2007), and toxic compounds produced by the wounding of cells are not all volatile (Pohnert 2004). For this reason, it is important to investigate if the information delivered by volatile compounds produced by diatoms is, in any way, related to the presence of toxic compounds in their cells, i.e., if there is any relationship between the effect of VOCs and the presence of other WCs in each given species of benthic diatoms. Since previous investigations demonstrated that some invertebrates recognize the VOCs produced by a benthic diatom (Jüttner et al. 2010), our main question here is whether the volatile infochemicals produced by each species of diatoms are related to the simultaneous presence of wound-activated toxins.

To test this hypothesis, we investigated the effects of WCs derived from three axenically cultivated strains of benthic diatoms isolated from the leaves of the seagrass *P. oceanica*. We tested their specific toxicity using a standard assay with sea urchin embryos (Romano et al. 2010). In parallel, we extracted volatile organic compounds (VOCs) from the same strains of diatoms and tested their infochemical activity on a set of invertebrates associated with *P. oceanica* using the protocol devised by Jüttner et al. (2010). In this study; however, the whole VOC assemblage extracted from three diatoms was used, not the bouquet of odours of *Cocconeis scutellum parva* (Grunow) Cleve, reproduced in the laboratory, as in Jüttner et al. (2010). This allowed us to test whether the level of toxicity characteristic of each species (due to various soluble toxicants, including WCs) is related to the presence of volatile infochemicals recognized by motile invertebrates.

**Materials and methods**

According to the main question of this investigation, our approach is composed of two distinct experimental procedures: (1) the extraction and test of volatile compounds (VOCs) to determine their possible role as infochemicals, from three species of diatoms and (2) the extraction and test of wound-activated compounds (WACs) from the same species of diatoms, to rank their specific toxicity by means of the standard sea urchin embryotoxicity test (Romano et al. 2010). The results obtained by means of the two methods were compared to assess whether a relationship exists between the behavioural effects triggered by volatile compounds and the presence of toxic metabolites in each diatom species.
This investigation is highly influenced by the dose of infochemicals administered, since a low amount of VOCs could be insufficient to produce reactions, while a high amount of VOCs could saturate the environment and disorder the invertebrates, in the experimental arena (Jüttner et al. 2010). Similarly, a high dose of toxic compounds could hamper the development of sea urchin embryos, but it could be out of the actual range occurring in the field (Jüttner 1999). In this case, an ecological role for these compounds could be negligible. For this reason, our experiments were conducted using an amount of diatoms that corresponds to ecologically relevant scenarios, e.g. a fish wounding a small portion of a P. oceanica leaf, or a group of invertebrates grazing on close areas over a leaf (Mazzella and Russo 1989). In particular, to test the effect of VOCs, we exposed various invertebrates to the equivalent of the diatoms contained in 64 mm² of P. oceanica leaves grazed off each minute (a total surface of 5,128 cm² of diatom film was divided into 400 agarose blocks, and the odour was diffused for 20 min). This amount is compatible with a natural range (Peduzzi 1987; Gacia et al. 2009), although several factors (e.g. temperature, currents, proximity to the origin) could modify the ecological effects (Fink 2007).

The problem is even more complex in the case of the toxicity tests, because the effect must be related to the biomass of grazers and, given the nature of the sea urchin test, it must be assayed in a liquid solution. For this reason, we tested a range of concentrations, corresponding (for each species of diatoms) to the wounding of cells covering 340, 170, 85, 17 and 1.7 cm² of P. oceanica surface area, respectively, extracted and administered per mL of body volume. This explains why the weights of diatoms tested on sea urchin embryos, according to the volume of medium, are different, because each diatom species reaches a specific biomass, when an available surface area is covered. As above, the ranges applied are according to ecologically plausible scenarios, where the highest concentration (340 cm² of equivalent leaf surface accumulated per mL of body volume of the grazer) corresponds to a long period of accumulation of toxins by a larger consumer (Havelange et al. 1997), while the lowest concentration (1.7 cm² of equivalent leaf surface per mL of body volume) may correspond to the hourly consumption of a small portion of diatom film by a medium size grazer (van Montfrans et al. 1982). Therefore, both toxicity and choice tests were performed in a range of concentrations having ecological implications.

Cultivation of diatoms and extraction of VOCs

Monoclonal cultures of three benthic diatoms were used for this study. Mother cultures of Cocconeis scutellum parva, Cocconeis posidoniae and Diploneis sp. have been maintained in a thermostatic chamber in f/2 medium at 18 °C with a 12:12 photoperiod. The light intensity ranged from 60 to 140 µmol photons m⁻² s⁻¹ according to the requirements of each species. These diatoms were cultured in 14-cm-diameter glass Petri dishes (Raniello et al. 2007) and moved to a −20 °C freezer after 15 days, when they covered almost evenly the glass surface. Forty Petri dishes for each of the above mentioned species were taken from the freezer and scraped thrice using a blade, in 5 mL of newly prepared culture medium. The total scraping time for all the plates was about 10 min, and the biomass of each diatom was re-suspended in 80 mL of filtered and sterilized seawater. To simulate the wounding of diatom cells, albeit they were already frozen and thawed (this process may generate breaks in the cells), each of the diatom suspensions was sonicated for 4 min and 5 mL of the suspension was taken for dry weight measurement, which was done by sieving the suspension through a pre-weighed glass fibre filter (GFF) via a syringe. Afterwards the filter was half folded, wrapped in aluminium foil and dried in the oven at 65 °C up to a constant weight. The weight of the collected diatoms for each mL of the suspension was calculated after drying. The dry weights of C. scutellum parva, C. posidoniae and Diploneis sp., added to each 80 mL of medium, to prepare the diatom suspensions used in our experiments, were 272, 144 and 254.4 mg, respectively.

Volatile organic compounds (VOCs) were extracted twice from the suspension (2 × 40 mL) for each species of diatoms. VOCs were concentrated by closed-loop stripping (Jüttner 1988) performed at 22 °C for 45 min. For this purpose, 40 mL of the sonicated diatom suspension was transferred to a 100-mL round-bottom flask and the VOCs extracted on a Tenax TA cartridge, after addition of 10 g NaCl (Fink et al. 2006a, b). After this time, most of the VOCs were adsorbed onto the Tenax cartridge; subsequently, the cartridge was removed and eluted with 6 mL diethyl ether. The ether was gradually evaporated using nitrogen (N₂, grade 5.0) gas, and the residue was redissolved in 300 µL of pure ethanol. Controls were prepared according to the same procedure, but stripping was performed on fresh f/2 (Sigma Guillard’s) seawater without the addition of any diatom. All VOC samples and controls were stored at −80 °C until the choice experiments were conducted.

Sea urchin embritotoxicity test

Toxic effects due to organic compounds (not necessarily volatile) produced by the same species of diatoms were detected by performing standard bioassays against sea urchin embryos. Adult sea urchins Paracentrotus lividus (Lamarck, 1816) were collected during the breeding season, in early spring, by SCUBA diving in the Gulf of

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Naples and transported in an insulated box to the laboratory, within 1 h after collection, and maintained in captivity in well-aerated water at 18 °C in an open circuit. Sperms and eggs were collected from at least three males and three females according to Romano et al. (2010). Eggs were fertilized and after checking for successful fertilization were incubated at a rate of 120 embryos mL\(^{-1}\) in filtered seawater at increasing concentration of diatom extracts. The bioassay was aimed at assessing if WACs produced by diatoms did exhibit any toxic effect on embryos, in order to compare these effects to the reactions of invertebrates detected by the odour choice experiments.

The three diatom species were collected and re-suspended in equal volumes of acetone (1:1), samples were centrifuged at 2,753 rcf for 6 min at 14 °C (DR15P B-Braun Biotech International), to separate the aqueous and the organic phases. At the end of the process, the resulting organic phases were combined and Na\(_2\)SO\(_4\) was added to dehydrate excess of aqueous phase if any, until it ceased to cake. The combined resulting organic phase was filtered into a round-bottom flask through a filter paper (Whatman 4). The solvent was removed in a rotary evaporator, and the residue was weighted and re-dissolved in methanol.

Therefore, sea urchin embryo toxicity tests were conducted, for each diatom species, on three different extracts, as above specified, viz: (1) “supernatant” (aqueous phase of the diatoms during the scraping process, possibly containing toxic compounds derived from the partial breakage of cells); (2) homogenate (aqueous extract of the diatoms after sonication); and (3) organic phase. The last two extracts could contain WACs derived from the classical pathways characterizing the activation of production of diatom secondary metabolites (Fontana et al. 2007b).

Fertilized eggs of the sea urchin P. lividus were incubated at different concentrations of each sample, and the embryonic development was followed under a Zeiss Axiovert 135 TV inverted microscope.

The concentrations in mg of dry weight for each diatom species, expressed as per mL of seawater, were different for Cocconeis scutellum parva, C. posidoniea and Diploneis sp., respectively, because they are referred to identical surface areas grazed, as explained above. Readings were taken 90 min, 24 h and 48 h after fertilization, but only the first and the last readings were considered in this study, for simplicity. In fact, the readings at 90 min revealed the percentage of cleavage and were used to assess an anti-mitotic activity while readings at 48 h revealed abnormalities and defects during the embryonic development. Fresh f/2 medium was used for negative controls.

Agarose preparation

To prepare 0.06 % agarose gel, 1.2 g of agarose (Sigma A-9045) was dissolved in 200 mL of filtered and sterilized seawater at 80 °C and stirred until completely transparent. The pH of the solution was adjusted to a value of 8.2–8.4 by adding 3.3 mL of 0.1 M NaOH. Controls were prepared by incorporating 250 µL of the control solution above described into still liquid (but close to room temperature) agarose, just before gelling. The agarose solution was then poured into a Petri dish and allowed to gel in a refrigerator at 5 °C, 1 h prior to the start of assays. To prepare VOC agarose blocks, 250 µL of the extracted VOCs were incorporated into the still liquid (but close to room temperature) agarose, just before gelling. Finally, the agarose disks were cut (using clean glass coverslips) into small blocks, each measuring 0.5 cm\(^3\) and used for the assays.
The choice tests

Twelve benthic invertebrate species were experimented for the odour choice assays (Table 1). Sampling was done using a plankton net (1 m frame diameter; mesh size 100-µm) trawled horizontally above a Posidonia oceanica meadow at Castello Aragonese (10 m depth), Ischia (40:43:52 N 13:57:55 E; Gulf of Naples, Italy). The invertebrates were sorted and identified in the laboratory and allowed to acclimatize 24 h in thermostatic chambers (18 °C, 12/12 photoperiod) prior to the start of experiments. The set of species chosen was based on the results of Jüttner et al. (2010), by selecting the invertebrates exhibiting the most interesting reactions to the VOCs produced by Cocconeis scutellum parva, with the addition of an omnivore (Caprella acanthifera), an omnivore-carnivore (Calculus tubularis), a herbivore (Rissio italiensis) and a detritus feeder (Bittium latreillii).

Odour choice experiments were carried out to demonstrate the extent of recognition of each diatom bouquet of VOCs by the selected set of invertebrates, inhabiting the same seagrass as the diatoms do. The assays were conducted in Petri dishes (14 cm diameter) positioned over a circular experimental arena printed on paper sheets (according to the protocols suggested by Jüttner et al. 2010). Each arena consisted of five sectors, viz: −2, −1, 0, 1, 2. These annotations refer to the distance from the positive target, being the sector +2 the one containing the agarose added with VOCs and the −2 the one containing the control agarose; “0” is the central sector, intermediate between the positive target and the negative control. Five individuals of each invertebrate species were released at the centre (marked as a circle) of each arena, and they were allowed to perceive the odour of the diatom, diffusing from the “+2” target. The number of individuals present in each sector of the arena was recorded at four time intervals (5, 10, 15 and 20 min) from the start of each test. Precautions were taken to minimize any external factor possibly influencing the movement of animals during the experiment, as light, temperature, magnetism, etc. As such, experiments were conducted at 18 °C under a well-lit and diffused light, and each replicated two arenas were positioned in such a way that the positive targets opposed each other. Six replicates were conducted for each invertebrate species versus each diatom.

Statistical analyses

As for the odour choice tests, the total number of individuals present in each sector during the whole experiment was calculated and a matrix “treatments versus arena sectors” was filled. We performed correspondence analysis (using the computer package STATISTICA version 10) on the above matrix consisting of five sectors (−2, −1, 0, 1, 2) and 36 treatments (12 invertebrate species × 3 diatom species), generating scores that indicated the main factors ruling our multivariate system. A bi-plot was drawn using the coordinates of variables (the sectors) and observations (the treatments), where different invertebrates are distributed in clouds and their proximity to the points representing the five sectors of the experimental arena indicate the degree of odour recognition exhibited by each species. Cluster analysis (using the package STATISTICA version 10) was carried out on the same matrix, in order to help spatial grouping of the coordinates yielded by correspondence analysis. This multivariate technique permits to detect the main factors ruling our ecological system, and it is the best suited to our multi-factorial data set. The interpretation of bi-plots is easy (Greenacre 2007), since the proximity of points in the same cloud indicates a close relationship and the contiguity of observations (the species tested) with the variables (the five sectors of our arenas) indicates that those given species are characterized by that degree of attraction or repulsion. The significance of the ordinations in the first
two dimensions was checked by means of the test proposed by Frontier (1974).

To compare the anti-mitotic potency (90 min) among three diatom extracts (supernatant, homogenate and organic phase) in the embryotoxicity tests and the significance of differences among diatom species hampering sea urchin embryonic development (48 h), we carried out a one-way ANOVA using GraphPad Prism 5 (GraphPad software). To compare the reactions of invertebrates towards the odour of three benthic diatoms, we calculated and plotted the average preference index (as proposed by Jüttner et al. 2010) exhibited by each tested invertebrate and the standard error. The latter was chosen according to previous authors (e.g. James et al. 2008) because the individual variability characterizing ethological responses is well known, and our objective was to show the average level of preference of each species, not the obvious scattering of results due to the natural random movements of invertebrates.

Results

Toxicity tests

The homogenate of *Cocconeis scutellum parva* did not exhibit any anti-mitotic effect since, even at the highest concentration, all sea urchin larvae exhibited total cleavage and normal divisions (Fig. 1a). After 48 h, however, only the lowest concentration produced an effect comparable with controls, since higher concentrations (from 750 to 2,700 µg mL^{-1}) gave a very low or null number of normal plutei (Fig. 1b). The congeneric *Cocconeis posidoniae* exhibited similar effects, besides the anti-mitotic activity observed at the highest concentration (Fig. 1c); after 48 h, concentrations higher than 100 µg mL^{-1} triggered a drastic reduction in normal plutei (Fig. 1d). A slightly higher toxicity was exhibited by *Diploneis* sp. (Table 2). In fact, even an intermediate concentration (1,000 µg mL^{-1}) induced...
arrest of the divisions at 90 min (Fig. 1e), and the embryo development was blocked in a pre-hatch phase at concentrations higher than 200 µg mL⁻¹ (Fig. 1f), in absence of normal pluteis.

As for the activity of organic extracts (Table 2), C. scutellum parva exhibited a very low anti-mitotic activity (Fig. 2a) since the first cleavage was retarded only at the highest concentrations. However, after 48 h, it showed clear developmental effects (Fig. 2b) at concentrations higher than 150 µg mL⁻¹. Cocconeis posidoniae exhibited, as well, a slight anti-mitotic activity (Fig. 2c) only at the highest concentrations of the extracts, as well as developmental effects (Fig. 2d) at high concentrations (more than 50 µg mL⁻¹). Diploneis sp. produced a maximum anti-mitotic activity (90 min; Fig. 2e) at the two highest concentrations (690 and 1,370 µg mL⁻¹), and embryotoxic effects after 48 h, at concentrations higher than 69 µg mL⁻¹ (Fig. 2f). In addition, all embryos were at the prism stage at 69 µg mL⁻¹ after 48 h, indicating a considerable hindering of development. The embryotoxic effects exhibited at the highest concentrations are comparable among the three diatoms.

In the case of the supernatant, we found negligible anti-mitotic effects (Fig. 3a, c, e) for all diatoms tested as the cleavage rate and the developmental effects were null or low. Nevertheless, C. scutellum parva and C. posidoniae supernatant exhibited a relevant effect on development, reducing at <10 % the percentage of normally developed plutei at a concentration of 550 and 250 µg mL⁻¹, respectively (Fig. 3b, d). Diploneis sp. exhibited a lower effect, confirming the very low toxicity of supernatants (Fig. 3f).

**Odour choice experiments**

The data recorded during the choice experiments performed on the VOCs from the three diatoms were ordered in a matrix “species versus sectors”, where the total number of individuals found in each sector of our experimental arena during each trial was reported. The correspondence analysis carried out on this matrix (Fig. 4) showed the presence of three main groups, as indicated by the cluster analysis. The ordination on the first two axes provides significant results according to the model proposed by Frontier (1974), and the first two dimensions yielded 50.64 and 22.06 % of the total inertia, respectively. The three main clusters identified were characterized by the presence of the sectors “0” (low level of odour recognition), “+2” and “−2” (maximum level of odour recognition) and “+1” and “−1” (intermediate level of odour recognition), respectively (Fig. 4). Therefore, the statistical technique suggests that the levels of attraction/repulsion, i.e. the levels of recognition of each diatom by various invertebrates, are the main structuring factors in the examined system.

In summary (Fig. 5), C. scutellum parva was characterized by an “intermediate” level of attraction/repulsion, since 6 species of invertebrates, i.e., Platynereis dumerilii, Hippolyte inermis, Cestopagurus timidus, Rissoa variabilis, Bittium latreillii and Gibbula umbilicaris, preferred the sectors “0” (weak recognition) and another three species, i.e., Dynamene bifida, Caprella acanthifera and Gammaridea fucicola preferred the sectors “+2” (strong recognition) as indicated by the correspondence analysis and the cluster analysis. A similar picture was obtained for the congenic Cocconeis posidoniae. In contrast, the tested invertebrates exhibited the lowest level of odour recognition for Diploneis sp., since six species, i.e., P. dumerilii, C. acanthifera, C. timidus, C. tubularis, R. italiensis and R. violacea, preferred the sectors “0” (weak recognition) and another three species, i.e., D. bifida, G. fucicola, H. inermis and R. variabilis, mainly moved to the sectors “+1/−1” and only two species were clustered with the sectors “+2/−2”. In particular, the latter two species are molluscs, i.e., B. latreillii and G. umbilicaris. Bittium latreillii exhibited a higher attraction for C. posidoniae and Diploneis sp. and a clear repulsion for C. scutellum parva (Fig. 6). In contrast, G. umbilicaris exhibited high attraction for C. posidoniae and repulsion for C. scutellum parva and Diploneis sp. As for the other

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Results of ANOVA analyses carried out on the main data set derived from the sea urchin embryo tests at 90 min and at 48 h, by comparing homogenates, organic extracts and supernatants to the negative controls.</th>
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<tbody>
<tr>
<td>90 min</td>
<td>Cocconeis scutellum parva</td>
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<tr>
<td>Homogenate</td>
<td>p &gt; 0.05</td>
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<tr>
<td>Organic extract</td>
<td>p &gt; 0.05</td>
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<tr>
<td>Supernatant</td>
<td>p &gt; 0.05</td>
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<tr>
<td>48 h</td>
<td>Cocconeis scutellum parva</td>
</tr>
<tr>
<td>Homogenate</td>
<td>p &lt; 0.0001</td>
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<tr>
<td>Organic extract</td>
<td>p &lt; 0.0001</td>
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<tr>
<td>Supernatant</td>
<td>p &lt; 0.0001</td>
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Asterisks refer to the results on the set of three species.
* Significance at p < 0.05;
** Significance at p < 0.01;
*** Significance at p < 0.001
species tested, *C. tubularis* and *G. fucicola* exhibited the highest levels of attraction towards *C. scutellum parva*, while *C. acanthifera*, *C. timidus* and *R. variabilis* exhibited the highest levels of repulsion towards *C. posidoniae*, *C. scutellum parva* and *Diploneis* sp., respectively (Fig. 6).

**Discussion**

The sea urchin assays yielded complex patterns of results from which some important concepts emerge. The two species of *Cocconeis*, at low and intermediate concentrations of homogenates and organic extracts, both showed no toxicity at 90 min (no anti-mitotic activity) and some developmental effects at 48 h, at lower concentrations. Similarly, the supernatants exhibited absence of toxicity even at the highest concentrations for all diatoms at 90 min. In contrast, we observed a clear influence on the larval development of all fractions, even at intermediate concentrations, at 48 h, for both species of *Cocconeis*. The absence of anti-mitotic activity is in agreement with previous investigations (Zupo 2000; Zupo and Messina 2006) indicating that *Cocconeis scutellum parva* is not toxic for benthic invertebrates. Nevertheless, it triggers specific physiologic reactions and induces the sex reversal in the shrimp *Hippolyte inermis* (Zupo et al. 2007). Here, we demonstrate that *Cocconeis* spp. produce polar compounds able to influence the embryo physiology and the larval development of sea urchins. These diatoms are very adhesive (*Cocconeis* spp. are much more adhesive than *Diploneis* sp.), and it is feasible that the process of detaching them from the glass surface caused disruption of several frustules and produced the
Fig. 3 Percentage of cleavage at 90 min (a, c, e) and percentage of normal embryonic stages at 48 h (b, d, f) recorded during the sea urchin embryo tests performed with supernatant of the diatoms Cocconeis scutellum parva (a, b), Cocconeis posidoniae (c, d) and Diploneis sp. (e, f), respectively.

Fig. 4 Correspondence analysis carried out on the matrix “index of preference versus species”. The three groups produced by cluster analysis are indicated in the space defined by the first two factors. Numbers refer to the species of invertebrates reported in Table 1. Letters refer to the three diatoms: S, Cocconeis scutellum parva; P, Cocconeis posidoniae; and D, Diploneis sp. Ellipses contain the species clustered around the five observations (five sectors of the experimental arena, from preference -2 to +2).
leaching of compounds. The toxicity cannot be due to exudates (Prince et al. 2010) or other constitutive compounds naturally released in the culture medium (Steigenberger et al. 2010), since the “supernatant” was produced using fresh culture medium, added to the diatoms during the process of detaching from the bottom of Petri dishes by means of blades. No other toxicity was present in the culture medium, as demonstrated by the negative controls.

*Diploneis* sp. exhibited a highly-specific toxicity (Table 2). The absence of toxicity of the supernatant may be explained taking into account that cells were not broken during the collection, because this species is scarcely adhesive. Therefore, the supernatant is equivalent to freshly prepared culture medium, not containing WACs. In contrast, its homogenates blocked the sea urchin cell divisions at 90 min, even at low concentrations, and arrested the embryonic development in a pre-hatch phase after 48 h, at concentrations as low as 10 µg mL⁻¹. The low anti-mitotic effect of its organic extract could indicate that WACs produced by this diatom have a hydrophilic character. In fact, the compounds produced after wounding are mainly concentrated into the aqueous solvents, and they are not collected by using non-polar organic solvents. All the organic extracts of the three diatoms, however, exhibited similar effects after 48 h, indicating the presence of bioactive compounds, while only the highest concentrations of the organic extract of *Diploneis* sp. exhibited cytotoxic effects at 90 min. We cannot exclude that the effects observed at 48 h with organic extracts and homogenates are influenced by deoxygenation, due to bacterial respiration, although the experiment was conducted at low temperature in order to reduce bacterial growth and assure a comfortable environment for sea urchin embryos.

It has to be remarked that toxicity tests were not performed with respect to the ecology of sea urchins, i.e., to demonstrate a natural toxicity of these diatoms that could influence the natural development of *Paracentrotus lividus* in nature. The planktonic larvae of this species, in fact, do not naturally feed on the benthic species of diatoms we considered in this study. We used the sea urchin embryo test as a routine tool for determining the presence of toxic compounds in our benthic diatoms, as suggested by previous authors (Pagano et al. 1986) and compare them with the level of odour recognition the invertebrates exhibited towards the same plant species (not necessarily due to the same compounds).

Our results indicate that the three diatoms have a different toxicity, with *Diploneis* sp. being the most toxic species, followed by *Cocconeis posidoniae* and *C. scutellum parva*, and that toxic compounds are mainly hydrophilic, since they are concentrated in the homogenates and present also in the supernatant (in the case of very adhesive species), but absent in the organic phase. This result is surprising, since most toxic wound-activated compounds known in diatoms are derived from the oxylipin pathway (Fontana et al. 2007b) and they are often represented by PUAs and...
their sectors, all characterized by a high lipophilicity (Wichard et al. 2005a). However, our comprehension of secondary metabolite toxicity is still incomplete and contrasting results were found, even in the well-studied relationships between PUA and planktonic copepods (Dutz et al. 2008; Wichard et al. 2008). Hence, we conclude that the sectors of our benthic diatoms, and especially of Diploneis sp., is due to scarcely known hydrophilic compounds (Lane et al. 2010), probably produced upon wounding and released in the water. In support of this hypothesis, it was demonstrated that several species of Bacillariophyceae do not produce PUA upon wounding when they are in the stationary growth phase (Wichard et al. 2005a; Vidoudez and Pohnert 2008), and our diatoms were collected at the end of their growth phase.

When we compare this pattern to the results of choice experiments, we find that invertebrates are ordered by statistical analyses mainly on the basis of their level of “odour recognition”, instead of a range of attraction/repulsion for the three diatoms by the considered species of invertebrates, as we could expect. This “odour recognition” is not developed for feeding purposes only (Jones and Flynn 2005) but also to use the information derived from the wounding of diatoms. Hence, invertebrates tend to recognize as infochemicals the diatom W. p. odour at a very high (−2/+ 2) or intermediate (−1/+ 1) level. In particular, Rissoa variabilis, B. latreillii, Platynereis dumerilii and other grazers were attracted by this diatom that, due to its low toxicity and the broad presence on P. oceanica leaves, may be a food source for various herbivores. However, R. italiensis, R. violacea and Cestopagus timidus exhibited very low recognition ability towards it. Cestopagus timidus is an omnivore–carnivore with scarce attitudes for the search of diatoms but, as a general trend, we observed that grazers living in the leaf stratum well recognized the “odour” of the two Cocconeis characterizing the epiphyte layer. For example, the meso-herbivore polychaete P. dumerilii exhibited a high level of recognition for C. scutellum parva (“1 S” in Fig. 4) and C. posidoniae odour (“1 P” in Fig. 4), but a negligible level of odour recognition for Diploneis sp. (1 D).

In conclusion, the two gastropod species that are most in contact with this diatom, sharing the same habitat, demonstrated the highest level of odour recognition. Other invertebrates exhibiting an intermediate level of recognition for Diploneis sp. odour are Dynamene bifida, Gammarella fucicola and Rissoa variabilis. These species are mainly found in the litter of P. oceanica, where Diploneis is more abundant than in the leaf stratum, due to lower washing out activities by the currents. In particular, G. fucicola is rarely found in the leaf stratum (Scipione et al. 1996), and it can be considered as a typical species of plant detritus accumulation (Gallmetzer et al. 2005), on which it feeds. It is also responsible for its fragmentation (Wittmann et al. 1981), but stable isotope analyses on ingested P. oceanica detritus showed a major trophic contribution by micro- and macro-epiphytes (Lepoint et al. 2006).

Among the other species present in the “non-recognition” sector, we observe that three invertebrates did not recognize the odour of C. scutellum parva and they are Calcinus tubularis, Rissoa italiensis and R. violacea. Calcinus tubularis is an omnivore hermit crab feeding mainly on animal prey, and it is probably scarcely attracted by diatom foods, but not repelled either. The two gastropod molluscs live mainly in the upper parts of the leaves, and they could have scarce opportunities to meet Diploneis sp.
toxicity of WACs produced by diatoms upon wounding. In fact, Diploneis sp., exhibiting the highest level of toxicity in the homogenates, is also the least recognized species by the investigated invertebrates, while C. scutellum parva, which is generally considered to be a high-quality food for several grazers with little toxigenic effects (Nappo et al. 2009), is well recognized by most species living in the leaf stratum of P. oceanica.

In conclusion, the toxicity of diatoms (Romano et al. 2003, 2010) was shown to be inversely correlated with the ability of animals to recognize their odours and this fits the scope of their insidious defence compounds (Miralto et al. 1999), but invertebrates tend to develop “good noses” for those species that are stable components of their environments, probably due to co-evolutionary processes (Fink 1999).

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**Centropages typicus** (Crustacea, Copepoda) reacts to volatile compounds produced by planktonic algae

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**Keywords**
Acidification; chemokinesis; copepod; diatom; planktonic; VOC.

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**Abstract**

Volatile organic compounds (VOCs) may play the role of infochemicals and trigger chemotaxis and ecologically relevant responses in freshwater and marine invertebrates. Aquatic grazers use these signals as chemical cues to trace the presence of their food or to detect their predators. However, detailed data are still needed to fully understand the role of these relationships in marine plankton. We investigated the ability of the copepod *Centropages typicus* to perceive the odour of three planktonic diatoms (*Skeletonema marinoi*, *Pseudonitzschia delicatissima* and *Chaetoceros affinis*) and a dinoflagellate (*Prorocentrum minimum*). This information is ecologically relevant for orientation, habitat selection, predator avoidance and communication. In addition, as the pH of the medium influences the perception of chemical cues in aquatic environments, we tested the effect of seawater acidification resulting from increasing levels of CO₂, and its influences on the olfactory reactions of copepods. For this reason, our tests were repeated in normal (pH 8.10) and acidified (pH 7.76) seawater in order to simulate future ocean acidification scenarios. Using replicated chemokinetic assays we demonstrated that VOCs produced by *Ps. delicatissima* and *Pr. minimum* attract copepods at normal pH, but this effect is lowered in acidified water. By contrast, the odour of *S. marinoi* mainly induces a reaction of repulsion, but in acidified water and at higher concentrations this toxic diatom becomes attractive for copepods. Our experiments demonstrate, for the first time, that copepods are sensitive to the volatile compounds contained in various microalgae; VOCs prompt chemokinesis according to algal species and odour concentrations. However, seawater acidification induces changes in copepods’ perception of odours. These findings highlight the sensitivity of chemically mediated interactions to global changes.

**Introduction**

A complex web of trophic, physiological and chemical relationships is shaped by the stable co-existence of plant and animal populations (Pohnert et al. 2007). Aquatic animals detect, discriminate and respond to a wealth of chemicals in their environment (Derby & Sorensen 2008). Information mediated by chemical cues between primary producers and their consumers may be associated with mechanical damage, herbivore presence or grazing activity (Verity 1991; Fink 2007; Van Donk et al. 2011). Various plant secondary metabolites are constitutive, i.e. constantly produced. Other metabolites are synthesized only in the presence of grazers (inducible or activated defences), as this reduces the maintenance costs compared with constitutive defences, improving their efficacy (Wolfe 2000; Amsler 2001).

Inducible defences in marine and freshwater phytoplankton and phytobenthos involve several classes of compounds with specific biological activity, many of
which are generated and/or liberated upon wounding processes (Pohnert 2002), released by a lipoxygenase cascade (Pohnert 2000; Jüttner 2005). Aldehydes, for example, synthesized by various planktonic diatoms (Pohnert 2002), produce deleterious effects on the embryonic development of their copepod grazers (Miralto et al. 1999) and inhibit mitotic proliferation in sea urchin embryos (Dutz et al. 2008; Romano et al. 2010). When planktonic diatoms are grazed, rapid onset of production of polyunsaturated aldehydes (PUAs) is observed a few seconds after the cell disruption (Pohnert 2000, 2005), similar to the wound reaction detected in higher plants (Blee 2002).

In general terms, phytoplankton shows induced changes in morphology, toxicity and life history, according to the species, and each microalga may use a range of strategies to discourage grazers (e.g. copepods, Vos et al. 2004; Daphnia, Carotenuto et al. 2005). Wound-activated compounds are also produced by benthic diatoms (Zupo 2000; Zupo et al. 2014) and freshwater algae, whose metabolites influence the activity of various grazers (Jüttner & Wurster 1984; Fink et al. 2006a,b). For example, 2(E),4(E),7(Z)-decatrienal has been shown to act as a repellent for freshwater crustacean grazers (Jüttner 2005).

Therefore, it is evident that wounded microalgae produce several metabolites that have a range of consequences for associated animals: deterrent for grazers (Ianora et al. 2008) or regulatory for animal populations (Zupo & Messina 2006), although the active compounds are generally different in benthos and plankton (Fenchel & Blackburn 1999; Zupo et al. 2007). Some of these secondary metabolites may be used by individual species as cues for finding food (Caldwell et al. 2002) or to obtain other fundamental information (Thomas 2010). In fact, various compounds carry biological information for invertebrates, playing the role of infochemicals (Dicke & Sabelis 1988), i.e. compounds bearing information that can be received by species living in the same environment. Chemoreception triggers chemokinesis and chemotaxis in various organisms in response to the presence of specific infochemicals (Breckels et al. 2011; Lewis et al. 2012).

Volatile organic compounds (VOCs) are amongst the fastest molecules in aquatic ecosystems and they may carry complex information (Wendel & Jüttner 1996; Jüttner & Dürr 1997; Fink 2007). These relationships have also been demonstrated in terrestrial ecosystems, where VOCs are named ‘odours’, as well as in benthic (Watson & Ridal 2004) and planktonic systems (Steinke et al. 2002). For this reason, in this study we refer to the effect of volatile compounds, to avoid the complex relationships with ‘tastes’ (e.g. amino acids, well known for their attractive power) and other compounds able to trigger a chemotactic reaction in various consumers.

Crustaceans rely on combinations of sophisticated chemosensory systems to identify and locate food, mates and predators in ‘noisy’ chemical environments suffused with a multitude of VOCs. They use antennular chemoreception to identify feasible food (Derby 2000; Derby et al. 2001) and locate it from a distance. Copepods may be attracted or repelled by given odours produced by microalgae, modifying their behaviour accordingly, as has been demonstrated in freshwater plankton (Durrer et al. 1999) and the marine benthos (Jüttner et al. 2010). However, little is known about infochemicals involved in the interaction between copepods and their plant prey (Van Donk et al. 2011; Heuschele & Selander 2014), although it has been demonstrated that various VOCs, such as dimethyl-sulphide (DMS), a biogenic gas derived from the algal secondary metabolite dimethyl-sulphopropionate, trigger search behaviour and chemotaxis in copepods (Steinke et al. 2006). Only recently have we begun to elucidate how chemical signals derived from biotic interactions have the potential to shape trophic relationships and complex community structures in aquatic systems (Klaschka 2009). These effects may also influence plankton ecology (Steinke et al. 2002; Nejstgaard et al. 2007).

Barofsky et al. (2010) demonstrated that the copepod Calanus sp. exhibits variable levels of ingestion of the diatom Skeletonema marinoi. Ingestion rates are higher during periods of high abundance (at the end of the exponential growth phase) whereas they decrease when the diatom is less abundant (at the beginning of the log phase). They hypothesized that these variable levels of preference could be because of infochemicals produced by the alga, although this was never confirmed. The present study checks the validity of their hypothesis by testing, in the laboratory, the effect of volatile infochemicals produced by this diatom, and the ability of a copepod to discriminate amongst various microalgal VOCs in normal and acidified conditions.

In fact, the seawater pH may influence the molecular assemblages of infochemicals (Hardege et al. 2011) as well as the ability of animal receptors (Jiang et al. 2002) to recognize them (Munday et al. 2010). Infochemicals synthesized by microalgae are produced and perceived in very low concentrations and their ecological activity may be sensitive to the presence of toxicants or to chemical and physical stressors, including ocean acidification and increased temperatures altering their production, degradation and perception (Vogt et al. 2008).

Therefore, seawater acidification, as forecasted for the coming decades, could influence the ecological relationships between planktonic invertebrates and their plant prey, as well as the stability of planktonic associations. Similarly, the ocean acidification predicted to occur over the next 100 years (Caldeira & Wickett 2005) could
influence the chemotaxis of planktonic organisms, with important ecological consequences for plant–animal interactions (Knutzen 1981), hampering the ability of copepods to recognize plant-generated chemical cues (Smith & Harper 2003).

Here, we investigated the effect of wound-activated VOCs produced by four planktonic microalgae on a widely distributed species of copepod, *Centropages typicus*, aiming at testing if (i) volatile infochemicals released by wounded algae are recognized as hypothesized by Barofsky *et al.* (2010) and trigger chemotactic responses because of attraction or repulsion, and (ii) if a decrease in seawater pH, as forecasted by Huesemann *et al.* (2002), will modify the strength and/or the direction of infochemical-mediated chemokinesis.

*Centropages typicus* was chosen because of its wide distribution (Ji *et al.* 2013) and its role in planktonic food webs (Kiørboe & Jiang 2013). This species is a good candidate for studies on the chemical interactions in plankton because of its ecological importance and physiological adaptations (Heuschele & Selander 2014). In addition, recent studies have been conducted on the effect of ocean acidification on its reproductive physiology (e.g. McConville *et al.* 2013).

**Material and Methods**

**Production of microalgae**

Four species of microalgae from the culture collection of Stazione Zoologica Anton Dohrn were used for the present investigation: *Skeletonema marinoi* strain FE66, *Pseudonitzschia delicatissima* strain 3668, *Chaetoceros affinis* strain FE21 and *Prorocentrum minimum* strain FE100. The first three are diatoms, often present in the phytoplankton blooms of Mediterranean coastal waters and normally found in the diet of planktonic copepods at various percentages. The fourth is an armoured marine planktonic bloom-forming dinoflagellate. Specific clones of this species may be toxic to humans (Ianora & Miralto 2010) but it is commonly administered as food to copepod cultures. It is cosmopolitan in temperate waters through to tropical regions, mostly estuarine but also neritic, actively swimming. *Skeletonema marinoi* is a colonial marine diatom responsible for winter maxima of phytoplankton blooms and produces aldehydes that negatively affect the embryonic development of various copepods (Ianora *et al.* 2004; Ianora & Miralto 2010). *Pseudonitzschia delicatissima* is another diatom producing biannual blooms in the Mediterranean. It is widely distributed, non-toxic (Maneiro *et al.* 2005) and generally present in the gut contents of copepods. Finally, *Ch. affinis* is a colonial diatom abundant in the Mediterranean Sea in spring and is important as a secondary prey for most copepod species.

To obtain a sufficient biomass for our experiments, multiple cultures of each strain were grown in 5-l carboys containing f/2 medium (Sigma Guillard’s f/2) at 18 °C. When the cultures reached their late exponential phase (6 days), 5 l were collected and concentrated by centrifugation (10 min at 1500 g at 4 °C). Each pellicle was then frozen at −20 °C until the extraction of odours.

**Extraction of odours**

To extract the odours produced after wounding, the frozen algal pellets (500 mg fresh weight for each algal species) were transferred into 100-ml round bottom flasks and activated by thawing in 25% NaCl (Fink *et al.* 2006a). The cells of each microalga were physiologically disintegrated, leading to the activation of the lipoxygenase cascade and the formation of volatile compounds (Pohnert 2002; Jütten 2005). Liberation of volatile compounds was also indicated by the appearance of a strong, rancid odour. The time needed for this procedure was about 10 min and it was sufficient to allow the production of VOCs. The VOCs were concentrated by closed-loop stripping (Jütten 1988) for 45 min at 22 °C, after which most of the VOCs were adsorbed on a Tenax TA cartridge. The Tenax-filled cartridge was removed, and the VOCs eluted with 6 ml diethyl ether, as described by Fink *et al.* (2006a), and the eluate collected in a clean glass tube. Subsequently, the ether was gently evaporated in a stream of nitrogen gas (N$_2$, grade 5.0) and the sample residue re-dissolved in 300 μl pure ethanol. The odours, re-solubilized in ethanol, were then dissolved in agarose gel at three concentrations (see below for preparation of gels) in order to simulate three different ecological scenarios. Additionally, control extracts were obtained using the same procedures on filtered seawater without algae. These extracts served as control treatments in the subsequent bioassays. All samples were stored in glass vials at −80 °C until used in the bioassays, in order to minimize the loss of volatiles.

**Sampling and rearing of animals**

Zooplankton was collected by means of a 200-μm Nansen net vertically towed from 50 m depth to the surface, at a coastal station (Station Marechiara: 40°48′ N, 14°15′ E) located in the Gulf of Naples. Samples were sorted in the laboratory to retrieve the model grazer, *Centropages typicus*. The selected animals were observed for their reactions to light, in order to check their fitness, then kept overnight in 2-l aerated glass vessels containing filtered (Millipore 0.22 μm) seawater until the start of the experiment. Animals selected for the experiment in acidified...
water were slowly acclimated to an intermediate pH (7.9), and then kept overnight in acidified water, in 2-l aerated glass vessels, up to the start of the choice tests. All animals were starved 12 h prior to the start of the experiments.

**Behavioural tests**

To test the effect of plant bouquets of odours on *Centropages typicus*, we conducted odour choice tests using a technique similar to the one proposed by Jüntner *et al.* (2010). Choice tests were conducted in order to distinguish positive and negative chemotaxis, mediated through the VOCs of each algal species (Wiesemeier *et al.* 2007). Differently from the study by Jüntner *et al.* (2010), our copepod essays were not performed in Petri dishes, but in elongated, elliptical Perspex containers with a minimum diameter of 13.0 cm and a maximum diameter of 27.5 cm and containing 400 ml seawater (approximately 2.5 cm water depth). The containers were placed in a climatic chamber at 18 °C with low light. This larger setup was chosen because of the relatively high mobility of copepods compared with the benthic invertebrates tested in the study by Jüntner *et al.* (2010). The shallow water depth ensured quasi-unidirectional diffusion of the odours in the experimental arena, avoiding complex diffusional effects that could bias the responses of animals and therefore hinder correct interpretation of the results. Calanoid copepods are strongly phototactic (Miljeteig *et al.* 2014) and performing the experiment in a low-light environment, with soft and well-diffused light, helped to minimize experimental bias (Stearns & Forward 1984).

Similar to the experimental setup by Jüntner *et al.* (2010), each arena contained: (i) two rectangles at the two opposite ends of the greater diameter, one for the odour sample and the other for the control, (ii) a central circle, used for the initial deployment of five copepod individuals, (iii) four vertical lines, delimiting five zones. The central zone was ranked zero (Fig. 1A and B). The two zones near the sample (+) were ranked 1 and 2, respectively. The two zones near the control (−) were ranked −1 and −2, respectively (Fig. 1A). The positions of (+) and (−) targets, in every two arenas, were opposed (Fig. 1C). Thus, external factors that might have influenced the movements of the animals, such as light, were randomized amongst the replicates, thereby excluding directional effects introduced by the experimental setup.

**Preparation of gels**

The agarose gels for tests and controls were prepared before the odour choice assays. We dissolved 150 mg of low-melt agarose (Sigma A 9045) in 25 ml of filtered and sterilized seawater at 80 °C and added 400 μl of 0.1 M NaOH to adjust the pH to a value of 8.2–8.4. The agarose solution was poured into a Petri dish and gelled in a refrigerator (5 °C) for 1 h prior to cutting the gel into blocks of 0.5 cm³. These gel blocks were then used as controls. For the experimental treatments, 2.5, 25 and 250 μl of the VOC bouquet obtained as described above were added to separate beakers, each containing 25 ml of agarose, while the bouquet was still liquid (but not hot, to minimize the evaporation of volatiles). Therefore, tests were performed for each alga at three different odour

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**Fig. 1.** Experimental arena used in the choice experiments: (A): different sectorial markings; (B): experimental arena; (C): Each experimental arena was positioned with the (+) target facing the (−) target of the next, to randomize the effect of light on phototactic organisms.
centrations: 0.01%, 0.1% and 1.0% by volume, corresponding to 0.17, 1.7 and 17 mg of each algae (FW) per ml of agarose, respectively. These concentrations, slowly diffusing into the experimental arena from 0.5 cm$^3$ blocks, correspond, approximately, to the wounding of microalgae naturally blooming in 1, 10 and 100 µl seawater, respectively.

After cutting the gels (using a clean glass coverslip), the odour-containing block of agarose was added to one edge of the experimental arena (+) and a block of control agarose was added to the other edge (−) as shown in Fig. 1B. The experiment started when five individuals of Centropages typicus were added to the central circle of each arena using a Pasteur pipette. The position of copepods (number of individuals present in each sector of the arena) was recorded after 5, 10, 15 and 20 min. All experiments were performed in seawater once at normal pH (8.10) and once in acidified water at pH 7.76. The acidified seawater was prepared in a 60-l tank by dosing gaseous CO$_2$ into the seawater using a Ferplast$^{TM}$ CO$_2$ distributor until the desired pH was reached, which was continuously checked with a temperature-compensated pH electrode pHep$^{TM}$ HANNA (HI 98128). After pouring the acidified seawater into the experimental arenas, its pH was checked again prior to the start of the experiment. Each of the four temporal records (5, 10, 15 and 20 min from the start of the experiment) was considered as a pseudo-replicate reading. The total number of individuals present in each sector, for each treatment, was finally computed.

Controls

Besides the control agarose present in each experimental arena, we performed a ‘double-control’ experiment, in order to check the movement reaction of copepods in the absence of odours. In particular, we devised a choice test with Centropages typicus exactly as explained above for the main tests. In this case, however, both targets were made of pure agarose gels without any odour (filtered seawater). Sixteen replicate tests in seawater at normal pH(8.10) were run in our arenas with control agarose in both directions. Our aim was to check if, in the absence of odours, the copepods exhibited any pattern of movement and any statistically significant difference in their spatial distribution. The data obtained by these tests were analysed as for the main data sets and the results were compared.

Control of the distribution of VOCs

The experimental arena and the position of the agarose blocks, as above specified, were set in order to produce a gradient of the VOCs concentration during the tests. However, in order to guarantee that volatile compounds were stratified according to the predictions, we performed additional measurements to track the distribution of odours in the arena. For this purpose, agarose blocks were charged with a known volatile compound (decadienal, an aldehyde produced by some planktonic diatoms upon wounding; Wichard et al. 2005). One hundred and fifty microlitres of decadienal dissolved in 4 ml of methanol was added to 200 ml of freshly prepared agarose, as described above. The mixture was then refrigerated and cut into 0.5 cm$^3$ blocks, as described above for the experimental VOCs, in order to give a final concentration of 240 µg : ml$^{-1}$. The blocks were then positioned in the (+) targets of the experimental arena, and the control blocks (agarose gels) were positioned in the (−) targets. Four sets of three replicate arenas were prepared contemporaneously. The vessels were positioned on a sheet of millimetric paper containing the indication of 30 randomly selected sampling points. A set of three replicates was sampled every 5 min by means of an automatic pipette, and 1 ml of the medium was collected, exactly in each sampling point on the grid. These collections were repeated at 5, 10, 15 and 20 min. The collected solution was then spectrophotometrically analysed (Hewlett Packard 8453 spectrophotometer) and the concentration of decadienal was calculated (Pippen & Nonaka 1958) taking into account its molar extinction (epsylon) in methanol, that is 31,000.

All concentrations obtained were recorded in a matrix indicating the position of samples and the averages of the three replicated samples at each time in each point were calculated. These values were used in the Kriging technique (Matheron 1969, 1970) that allows for a spatial representation of the concentration, considered as a stationary phenomenon. In this way we obtained a map of the concentrations at each point of the experimental arenas, described by isolines, suitable to confirm the exactness of our hypotheses and track the diffusional gradient from the agarose gels in the elliptical containers.

Statistical analyses of results

The number of individuals in each sector during each treatment was recorded. The mean number (±SD) of individuals present in each sector, for six replicates, was calculated for each species and time interval (5, 10, 15 and 20 min). The significance of the differences in the distribution of individuals between the control areas (−2, −1) and the VOC areas (+2, +1) for each species in the four time periods was tested by repeated-measures one-way analysis of variance (ANOVA) using GRAPHPAD PRISM 4 (GraphPad software, La Jolla, CA, USA). The same analyses were
performed for the control experiment. In the case of *Skel-
etonema marinoi*, to check the validity of the hypothesis by
Barofsky *et al.* (2010) suggesting that infochemicals may
influence the variable levels of feeding on this alga by
copepods, as observed at various concentrations, we used
the index proposed by Jüttner *et al.* (2010) to evaluate
the preferences of copepods at each concentration. This index
summarizes the data obtained in the positive and negative
sectors, yielding a score between −4 (total repulsion) and
+4 (total attraction), passing through zero (scarce or null
activity of the VOCs).

A matrix was filled, which contained five sectors (from
−2 to +2) and 24 treatments (four algal species ×
three concentrations × two pH). A Correspondence
Analysis (CA) (using STATISTICA 10, Statsoft, Tulsa, OK, USA)
was performed on this matrix and the first two factors
were plotted in order to detect the main ‘preferences’
deriving from each treatment according to the proximity
to given sectors. The picture obtained by the CA was
further interpreted by performing a Cluster Analysis
(using STATISTICA 10) on the same matrix in order to
help the identification of homogeneous clouds in the bi-
plot. The significance of axes was tested by means of the
‘broken-sticks’ model (Frontier 1974).

Results

Kriging analysis of the diffusion

The Kriging analysis of the decadienal concentrations,
measured every 5 min, confirmed our hypotheses about
the diffusional trends of VOCs into the experimental are-
enas (Fig. 2). In particular, at the start of the experiment a
clear gradient of concentration was observed (Fig. 2A)
and it persisted during the experiment (the intermediate
plots have been omitted for simplicity) until the end
(Fig. 2B). In fact, at 20 min a clear gradient was still
present, although less pronounced, from the (+) target
towards the (−) target.

Bioassays

The most striking reactions were exhibited by copepods
under *Prorocentrum minimum* treatments at lower con-
centrations (Table 1), as indicated by the significant dif-
fferences of the ANOVA (Table 2) and the cluster
analysis. At the lowest concentration, VOCs from this
species were highly attractive to *Centropages typicus* at
both pH values. At intermediate concentrations, the

![Fig. 2. Kriging reconstruction of the
distribution of a volatile compound
(decadienal) at (A): 5 and (B): 20 min after
the start of the experiment. Isolines indicate
areas with identical concentrations of the
volatile organic compounds according to the
intensity scale on the right (µg · ml⁻¹).](image-url)
bouquet was attractive at normal pH (8.1) but not in acidified water (Fig. 3A). At the highest concentration it had no effect and the ANOVA analysis also indicated the absence of significant differences between the positive and the negative sectors.

*Pseudonitzschia delicatissima* was active as well, but only at the lowest concentration and at normal pH (Fig. 3B). In fact, ANOVA indicated the absence of significant differences at the acidified pH at the lowest concentration. At higher concentrations *Ps. delicatissima* had no effect (*P* > 0.05; Tables 2 and 3). VOCs released from wounded cells of *Chaetoceros affinis* triggered complex patterns of behavioural responses in *Ce. typicus* under various conditions and the results of the ANOVA (Table 2) indicated that they had no clear overall effect, either attractive or repulsive, on our model species (Fig. 3C).

Finally, the behaviour of *Skeletonema marinoi* was totally independent of the pH. No significant differences were detected between the two treatments at normal and low pH for any of the three concentrations (ANOVA; *P* > 0.05). Most behavioural patterns, indeed, are perfectly reproduced at low and normal pH (Fig. 3D). *Skeletonema marinoi* produced a repellent effect at low concentration, an attractive effect at the intermediate concentration and no effect (absence of significant differences) at the highest concentration (Tables 2 and 3).

To answer the main questions of our study as stated above, the preference indices proposed by Jüttner *et al.* (2010) were calculated for *S. marinoi*. These indicated that this alga is highly repulsive to copepods at low concentrations, highly attractive at intermediate concentrations and has negligible effects at the highest concentration (Fig. 4).

The control experiment, performed in 16 arenas containing two negative targets, showed no significant differences in the distribution of *Ce. typicus* (ANOVA; *P* = 0.12; Table 2). In this case, the space distribution of copepods was random, even when single time points were analysed (non-significant differences in the spatial distribution of copepods at 5, 10, 15 and 20 min).

These data are summarized by the correspondence analysis (Fig. 5), indicating three main clouds of reactions, defined by the proximity to sectors +1 and +2 of the experimental arena (positive responses) in the bottom right of the diagram, 0 (absence of reaction) in the top right of the diagram, and −1 and −2 (negative response) in the top left of the diagram. Two treatments (*Ps. delicatissima* at low pH and medium concentration, and

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**Table 2.** Results of the ANOVA analyses performed on the matrix of the number of individuals present in the sectors $-2$, $-1$, $+1$ and $+2$ of each treatment. L, M and H indicate volatile organic compound concentrations 'low', 'medium' and 'high', respectively, as indicated in methods. The last row (0) refers to the control tests (16 replicates), in which both targets contained a blank agarose block. The values of Fisher’s F and P are reported for each treatment.

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<td>0.91</td>
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<tr>
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<td>CTRL</td>
<td>pure agarose blocks in both targets</td>
<td>1.83</td>
<td>0.12</td>
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</table>

**Pr. minimum** at high concentration and low pH do not fall within this scheme and produce a separate cluster in the lower left part of the diagram.

The cluster analysis confirmed the three main clouds detected by CA (Fig. 6). The results of the CA are highly significant, as indicated by the application of the ‘broken
Table 3. Summary of the results obtained, taking into account the significance of differences indicated by ANOVA (Table 2) and the four clusters found by the cluster analysis (Fig. 6), ordered by means of the correspondence analysis (Fig. 5). ‘+’ indicates attraction; ‘−’ indicates repulsion; ‘0’ indicates absence of reaction by the copepod.

<table>
<thead>
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<th>conclusion</th>
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</tr>
<tr>
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<td>6</td>
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<td></td>
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<tr>
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<td>+</td>
<td>Pseudonitzschia delicatissima 8.1</td>
<td>attractive at low concentration.</td>
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<td>Different effects according to pH</td>
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<td>0</td>
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Fig. 4. Preference indices (according to Jüttner et al. 2010) for Centropages typicus responding to volatile organic compounds from Skeletonema marinoi at the two pH values and the three concentrations (low, medium, high) tested.

Fig. 5. Preference indices for Centropages typicus chemotaxis to Skeletonema marinoi VOCs.

Discussion

The chemical milieu of aquatic environments shapes how animals perceive their world (Derby & Sorensen 2008). At present, according to Fink (2007), our knowledge of the identity of ecologically relevant chemicals is limited to a few contexts (especially feeding) and a few species, often commercially important ones. The results of this investigation indicate that the model copepod used reacts in different ways to a range of microalgal odoriferous products. Consistent results were obtained at different pHs with VOCs extracted from Skeletonema marinoi. The compounds produced by Pseudonitzschia delicatissima and the dinoflagellate tested in this study are less stable and their chemical structure may change according to the pH of the medium (Wikfors 2005; Barreiro et al. 2011).

Food selection and ingestion in crustaceans are influenced by a blend of attractive and deterrent compounds, although we know more about the former than the latter (Derby et al. 2001; Prusak et al. 2005; Kamio et al. 2007).
In our case, the nature of the VOCs extracted is not known because our aim here was to detect the reactions of copepods exposed to the whole bouquet of VOCs produced by the wounding of planktonic microalgae.

In addition, the actual concentration of VOCs in the solvent used here is not known, but we do know that the VOCs dissolved in our experimental arenas corresponded to the amount of microalgae naturally blooming in 1, 10 and 100 μl of seawater, according to the methods applied. The range of results obtained at various concentrations may be explained according to the arguments reported by Jüttner et al. (2010). Even when odour concentration is low, many species of marine invertebrates can easily detect its origin and, in the case of infochem-
icals, they can ‘decide’ to move towards or away from the source of VOCs, according to the message received. Some species of benthic invertebrates, for example, might choose to move away from a diatom odour because this indicates the presence of possible predators that may damage the diatoms and their physical substrate (Jüttner et al. 2010). Similar reactions have been found in terrestrial ecosystems (Evans 1982).

VOCs extracted from *S. marinoi* induced an escape reaction at low concentration in our arenas, and this is in agreement with decreased feeding observed by Barošky et al. (2010) in mesocosms, at low densities. This may be explained by the fact that *S. marinoi* is a potentially poisonous diatom that induces teratogenic effects in copepod embryos (Miralto et al. 1999). At the highest concentration used in the present study this alga became attractive (consistent with the conclusions reported by Barošky et al. 2010) possibly because of the effect of other odoriferous compounds that become ‘interesting’ at higher concentrations (Taylor et al. 2009). The adaptive value of this behaviour and its ecological implications remain unexplained but it is known that *Centropages typicus* actively ingests this diatom, especially when it is abundant in the water column (Amin et al. 2011).

*Centropages typicus* feeds on *S. marinoi* during bloom periods (Jonasdottir 1994; Jonasdottir et al. 2009), whereas its ingestion is reduced when it is less abundant. In fact, at the low concentration of the odour the diatom induced repulsion, whereas at the medium concentration it clearly mediated an attraction. These reactions were reproduced at both pH regimes. When the concentration increased further, no behavioural response of *Ce. typicus* was observed. This indicates that the environment of the experimental arena might have been saturated by the diatom odour and therefore that there was no clear odour gradient available for the copepods to make decisions (Jüttner et al. 2010). As reported above, these effects were highly significant and independent of the water pH, which indicates that the VOCs relevant for this interaction are not influenced by acidification. Therefore, the reactions of *Ce. typicus* to the odour of *S. marinoi*, as shown by our experiments, are in agreement with those previously observed in mesocosms and in the laboratory (Barošky et al. 2010). However, previous authors hypothesized that the contrasting reactions could be the result of variable production of infochemicals during the life cycle, and seasonal variability in the production of aldehydes has been demonstrated in *S. marinoi* (Taylor et al. 2009). By contrast, our experiments indicate that the same VOCs, at various concentrations, are responsible for the copepod’s reactions. Copepods are attracted by this diatom when they perceive higher concentrations of VOCs.

By contrast, *Ps. delicatissima* is considered to be a nontoxic diatom (Maneiro et al. 2005) and it produces some VOCs that were clearly distinguished by our model copepod at the lowest concentrations used here, triggering a rapid attraction (Table 3). These compounds (or the sensing of them by copepods) are evidently influenced by pH because the attraction disappeared in acidified seawater. At higher concentrations odour saturation was reached immediately, probably because of the high sensitivity of copepods to the infochemicals present in the odour bouquet of this species; *Ce. typicus* showed indifference to the odour at medium and higher concentrations. This indicates that specific compounds are easily recognized by copepods, that they act at low concentrations and that their chemical structure and/or their recognition by copepods are pH-dependent. This result explains the frequent presence of *Ps. delicatissima* in the gut contents of *Ce. typicus* (Carotenuto et al. 2006), which considers it, evidently, a good prey. Hence, some infochemicals are recognized even at a low concentration, when microalgal cells are broken by various grazers, triggering a rapid attraction response in the copepods.

The dinoflagellate *Prorocentrum minimum* is considered to be a high-quality food item for *Ce. typicus* (Calbet et al. 2007). In fact, it is currently administered to laboratory cultures of this copepod, inducing high percentages of survivorship (Buttino et al. 2012) and viable embryos. It is therefore not surprising that it was recognized by and attracted *Ce. typicus* at both the lowest and medium concentrations. The well-known saturation effect (Jüttner et al. 2010) was observed at the highest concentration, consistent with the results obtained for the other algae. In this species, however, the effect of pH was negligible at the lowest concentration, whereas it was evident at the medium concentration. Some VOCs produced by this dinoflagellate are relatively stable in seawater, but are different from those produced by *S. marinoi*.

By contrast, VOCs extracted from *Ch. affinis* triggered no reaction in our model species. It is also considered to be a possible prey for *Ce. typicus*, although it is not highly preferred (Belgrano et al. 2004; Bandelj et al. 2008). The results of the present study indicate that it does not produce important infochemicals or, at least, that the VOCs produced upon wounding of this species are not recognized as infochemicals by *Ce. typicus*.

These results confirm our hypotheses and those of previous authors (Vardi 2008) that the wounding of some planktonic algae produces bouquets of odorous compounds recognized by copepods and that trigger specific reactions. In general, VOCs released upon the wounding of these algae trigger an attraction, except for *S. marinoi*, which induces an escape reaction at low concentrations. This evidence indicates that the presence of algae repre-
senting possible foods is a primary cue for planktonic copepods such as *Ce. typicus*. In fact, feeding cues primed the effects of some of the most important infochemicals for crustaceans (Weissburg & Zimmer-Faust 1991; Moore et al. 1999; Archdale & Anraku 2005). The reactions of the copepods were statistically significant in these experiments whereas no significant differences in their spatial distribution were observed in the control experiment, which tested two negative controls. This demonstrates that the technique applied allows for a clear indication of the odour preferences in copepods and that their spatial distribution is a response to the VOC gradient provided by the presence of the positive target in the experimental arena.

Our results demonstrate that infochemicals may transmit complex messages to copepods. Large differences exist in the production of total PUAs (sum of heptadienal, octadienal, and octatrienal) amongst *S. marinoi* strains isolated at different times of the year (Barofsky et al. 2009). In particular, diatom strains sampled in different seasons exhibited complex patterns of PUA production, according to the growth cycle, with summer strains having consistently high PUA production potential in both exponential and stationary growth phases in comparison to other strains, whereas spring strains exhibited a low PUA production during the exponential growth phase (Taylor et al. 2009). However, grazing pressure by copepods is much higher during summer and autumn than in spring, becoming a significant threat to microalgal communities (Lindahl & Hernroth 1983; Vargas et al. 2002). Copepods have been shown to ‘prefer’ *S. marinoi* when it is more abundant and to avoid it when it is scarce (we observed an escape reaction at low abundances). Diatoms protect themselves in periods of higher grazing by producing more PUAs (Tonnesson et al. 2005). These relationships may also be explained by taking into account that chemoreceptor neurones of crustaceans enable discrimination of highly similar mixtures, such as binary mixtures of 30 components that contain the same compounds at different blend ratios (Steullet & Derby 1997; Derby 2000; Derby et al. 2001). Variable concentrations of these blends may produce contrasting effects on the same species of copepod, as we have demonstrated in this study.

As specified above, we did not quantify the concentration of the VOCs tested in this study as they were not individually analysed but extracted from known amounts of planktonic microalgae derived from laboratory cultures. However, for all of four species of microalgae tested, we obtained consistent results according to the concentration, *i.e.* the volatile compounds were recognized at the lowest and intermediate concentration (and the two concentrations could trigger contrasting reactions, in agreement with the findings by Barofsky et al. 2010), whereas at the highest concentration the VOCs did not produce active reactions, probably because of saturation (Jütterm et al. 2010). This observation indicates that the odours of most microalgae are easily detected by copepods at very low concentrations, even over long distances (DeMott & Watson 1990) and that chemoreception may be an important sense for distance detection of possible prey and recognition of toxic algae.

Finally, this investigation has revealed that the recognition of some of the infochemicals produced by the wounding of individual planktonic microalgae is influenced by the water pH and that a pH change comparable to the one forecasted for the year 2100 (Caldeira & Wickett 2005) may result in dramatic alterations in the specific reactions of copepods. For example, medium concentrations of the ‘good’ alga *Pr. minimum* should attract copepods, but at a low pH they act as a deterrent. As a consequence, the feeding ethology of this and other species of copepods could change, resulting in mortality and reduction of natural stocks because of impairment to the infochemical network of information coupled with a direct effect on some species of copepods.

Rising atmospheric carbon dioxide concentration is causing global warming and ocean acidification (Caldeira & Wickett 2003; Feely et al. 2004; Orr et al. 2005), which are recognized to be important drivers of change in biological systems (Lovejoy & Hannah 2005). Kurihara et al. (2004) demonstrated a decrease in egg hatching success in the copepod *Acartia staueri* because of a lowering of the water pH, which indicates that such changes in water chemistry may adversely impact natural populations and the whole equilibrium of oceanic communities. In addition, the two-first nauplii stages of the boreal calanoid copepod *Calanus finmarchicus* (a key species for planktonic food webs) may be vulnerable in a more acidic ocean (Pedersen et al. 2014), although this species may be considered robust to direct effects of ocean acidification.

Therefore, it is very likely that changes in ocean pH may influence both the molecular shape of some important infochemicals and the ability of animal receptors to recognize them. A critical need arises to test the physiological consequences of ocean acidification at various pCO$_2$ levels, such as those forecasted to occur over the next century. Our research has demonstrated that a low pH dramatically interferes with the effect of plant-produced infochemicals on a common copepod and, in the case of such species of diatoms as *Ps. delicatissima* and *Ch. affinis*, it triggers an inversion of the natural reactions of attraction or escape of the grazer to their wound-activated odours. Other blends of odours, by contrast, such as those produced by the diatom *S. marinoi*, were not influenced by the pH under our experimental conditions.
Conclusions

The need for further investigations clearly emerges, as our understanding of how crustaceans process sex pheromones, social cues, anti-feeding compounds and other chemical stimuli, besides feeding stimulants and mecanoreception, is still in its infancy (Huesemann et al. 2002; Heuschele & Selander 2014). The results reported here indicate that wound-activated infochemicals, previously demonstrated to be important in terrestrial and benthic systems, are also essential cues for a planktonic copepod and may contribute to the stability of food webs and carbon cycling through bottom-up controls, involving pH-dependent bioactivity of volatile metabolites.

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Chapter 3.7. Trophic resources may be predictable and influence biodiversity: a general model

Summary

3.7.1. Relating trophic resources to community structure: a predictive index of food availability. (Cited by 5. I.F.= 2.51)
The abundance and the distribution of trophic resources available for consumers influence the productivity and the diversity of natural communities. Here an index of food abundance has been proposed, the framework of which can be adapted for different ecosystems. The relative available food index (RAFI) is computed by considering standard resource conditions of a habitat and the influence of various generalized anthropogenic and natural factors. RAFI tables can be applied to a range of marine ecosystems for predictions of the potential abundance of food available for each trophic group. They will be critical to demonstrate the validity of the general model relating the availability of feeding resources to the biodiversity of ecosystems.
Relating trophic resources to community structure: a predictive index of food availability

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The abundance and the distribution of trophic resources available for consumers influence the productivity and the diversity of natural communities. Nevertheless, assessment of the actual abundance of food items available for individual trophic groups has been constrained by differences in methods and metrics used by various authors. Here we develop an index of food abundance, the framework of which can be adapted for different ecosystems. The relative available food index (RAFI) is computed by considering standard resource conditions of a habitat and the influence of various generalized anthropogenic and natural factors. RAFI was developed using published literature on food abundance and validated by comparison of predictions versus observed trophic resources across various marine sites. RAFI tables here proposed can be applied to a range of marine ecosystems for predictions of the potential abundance of food available for each trophic group, hence permitting exploration of ecological theories by focusing on the deviation from the observed to the expected.

1. Introduction

1.1. The importance of trophic resources

Nutrient supply and productivity gradients can strongly influence the diversity of natural communities through trophic linkages.
[1,2]. Consequently, attempts to predict biodiversity patterns in marine ecosystems should consider the abundance of food available for different trophic groups [3,4]. To date, research has been focused primarily on influences of predators on prey populations, through a top-down approach [5]. Various studies also suggest that resources and consumers interact to structure food webs [6,7] with, for example, demonstration that herbivore and predator abundances vary predictably along natural productivity gradients [1].

Unfortunately, the various forms of trophic data reported among studies impede broad-scale comparisons because of different sampling methods, different trophic groups, incomplete sets of plant and animal taxa, and different units of measurements [8,9]. In the marine context, benthic and planktonic morphofunctional groups are often sampled with different instruments, on different surface areas or volumes, and among different habitats. For this reason, only a few broad-scale cross-ecosystem comparisons have yet been made on relationships between productivity/functioning and food resources available for each trophic group [3,5].

1.2. Prediction of trophic resources

Nevertheless, a classification of ecosystems based on the abundance of each trophic resource is theoretically possible [10]. For example, the amount of plant biomass potentially available for macroherbivores will inevitably be much higher in seagrass meadows than unvegetated sandy substrata or marine caves [11]. In addition, the abundance of food available for macrocarnivores is higher on coral reefs than shallow seaweed meadows [12,13]. Extending such generalizations, food resources available to different trophic groups can be evaluated by considering habitat constraints.

Various pressures acting locally also influence and modulate these general trends. For example, the abundance of plant detritus is high in seagrass meadows, but the presence of strong currents may disperse the detritus particles and make that resource less abundant [14]. Wave exposure and associated surge also negatively influence detritus, potentially reducing availability for herbivore–detritivores. Additionally, food for microherbivores is abundant in shallow rocky bottoms and increases with increasing nutrients [15], but declines in deep rocky environments, owing to the limiting influence of light [16]. Therefore, nutrient availability and depth are important moderating factors, with consistent effects across a range of ecosystems [17].

Our study aims at describing general patterns of relative abundance of food available for trophic groups among various marine habitats. Based on these patterns, we developed a mechanistic model of food availability and validated its predictions through comparisons of computed versus observed food resources at several comprehensively sampled sites. Trophic resources were assessed solely on the basis of their physical presence in each habitat, irrespective of whether the food material was protected by physical, chemical or behavioural defences [18]. The model is presented here in order to easily incorporate an estimate of trophic resources in evaluations of diversity–productivity relationships [19] and in other analyses of marine ecosystems.

2. Material and methods

2.1. Computation of relative available food index tables

The relative available food index (RAFI) was computed by screening the global literature on trophic resources in marine habitats (electronic supplementary material, table S1). A literature search was conducted using ISI Web of Science™ (www.webofknowledge.com) from 1945 to 2010, plus hardcopy literature contained in the library of Stazione Zoologica, Naples, that encompasses magazine collections from 1872 to the present. Studies involving abundance and taxonomic composition of marine organisms were considered when the information contained was comparable and appropriate, in terms of surface units, abundance units, substrata and taxonomic groups investigated. Restrictions related to language, publication date or publication status were not imposed. The data recorded show regional patchiness, owing to the availability of specific studies according to the distribution patterns of authors (table 1). The first step was the evaluation of the food resources available at each of five substrata (hard, soft, hard biogenic, macroalgae and seagrass beds; table 2) and for 11 trophic groups (table 3) that were expressed according to the type and size of prey items [20].

To calculate the abundance of food potentially available for microcarnivore (mCa) species in each substratum, for example, research articles containing data on the abundance of microcarnivore prey (meiofauna and other small animals less than 1 mm in size) were selected, and the abundances reported...
Each ecosystem was consequently classified according to the amount of food potentially available to each trophic group (tg), according to the following relationship:

$$\text{Resource abundance}_{(tg, \text{ecosystem})} = f(\text{basic substratum} \times \text{specific habitat})$$  \hspace{1cm} (2.1)

This permits estimation, for example, that the plant standing stock potentially available for herbivores (He) is maximum in a fucofoid or a seagrass meadow, lower in harbours and lowest on sandy substrata, coral reefs and caves (table 4a).

### Table 1. Geographical distribution of the studies used for the construction of the RAFI model. The number of publications considered for each region is reported in columns, according to various ecosystems (resulting from the classification in electronic supplementary material, table S1), in rows. The total per cent contribution of researches performed in each region is reported in the last row.

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</table>

### Table 2. Example of the ranking process applied to herbivore (He) food resources for five substrata (in rows). A score from 1 to 3 is attributed (third column) according to the ranges of abundance reported (second column). The literature used to obtain abundance ranges is indicated in the fourth column (numbers in brackets are referred to electronic supplementary material, table S1).

<table>
<thead>
<tr>
<th>basic substrata</th>
<th>abundance of food for He</th>
<th>rank (1–3)</th>
<th>literature</th>
</tr>
</thead>
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<tr>
<td>soft substratum</td>
<td>0.3–6 g C m$^{-2}$</td>
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<td>56–234 g C m$^{-2}$</td>
<td>2</td>
<td>(26,30,34)</td>
</tr>
<tr>
<td>hard biogenic</td>
<td>41–140 g C m$^{-2}$</td>
<td>2</td>
<td>(21,22)</td>
</tr>
<tr>
<td>macroalgae beds</td>
<td>40–310 g C m$^{-2}$</td>
<td>3</td>
<td>(35,38,42)</td>
</tr>
<tr>
<td>seagrass beds</td>
<td>20–600 g C m$^{-2}$</td>
<td>3</td>
<td>(47,54,75,76)</td>
</tr>
</tbody>
</table>

by different authors (in various sites, seasons, etc.) were recorded. Similarly, to evaluate the abundance of food available for macroherbivores (He) in various substrata, papers containing information on the standing crops of plants and algae were selected for each of five habitats, and abundance data were recorded (table 2, second column). Available data may be expressed in several different units (e.g. number of individuals, mg of biomass, μg of carbon or kcal per unit surface area) according to the methods followed by each author. In these cases, all data were converted, according to [21], to g C m$^{-2}$, in order to permit comparisons among the different studies. Finally, the range of abundances recorded (figure 1) was divided into three intervals ranked 1 (low abundance), 2 (medium) and 3 (high), as indicated in table 2 (third column). The interval subdivision was made according to a best professional judgement in order to highlight the differences found among ranges.

Subsequently, each basic substratum (table 3a) was further divided into specific habitats (table 3b), based on the distinctions made in most trophic models [22] and each food category was assigned to an abundance interval (1–3), for each of 10 specific habitats (table 3b and figure 2), as described above. For example, hard substrata were grossly divided into rocky reefs and coves, according to the different exposures to light and external influences characterizing these environments. Similarly, soft substrata were divided into open sand and embayments, based on variable shelter influencing plant and animal communities (table 3b).

Each ecosystem was consequently classified according to the amount of food potentially available to each trophic group (tg), according to the following relationship:

$$\text{Resource abundance}_{(tg, \text{ecosystem})} = f(\text{basic substratum} \times \text{specific habitat})$$  \hspace{1cm} (2.1)
Finally, modifying factors were considered, to explain how local environmental conditions influence the food resources available for a particular trophic group with respect to the average conditions for a given habitat. These modifiers acknowledge that other factors, besides the type of substratum and the specific habitats, influence the community composition and the abundance of trophic resources [20,23,24]. For example, variations of light irradiance owing to depth may dramatically influence the abundance of plant biomass present in a deep rocky reef or seagrass meadow. The
conditions. Trophic groups: mCa (microcarnivores); Ca (carnivores), mHe (microherbivores), He (herbivores), mOm (microomnivores), Om (omnivores), mDeF (microdetritus feeders), DeF (detritus feeders), DeFS (detritus feeders—suspensivores), DeFHe (detritus feeders—herbivores) and FF (filter feeders).

Table 4. (a) Final scores with RAFI predictions for average abundances of trophic resources in each habitat. (b) Modifiers for local conditions. Trophic groups: mCa (microcarnivores); Ca (carnivores), mHe (microherbivores), He (herbivores), mOm (microomnivores), Om (omnivores), mDeF (microdetritus feeders), DeF (detritus feeders), DeFS (detritus feeders—suspensivores), DeFHe (detritus feeders—herbivores) and FF (filter feeders).

![Diagram](http://rsos.royalsocietypublishing.org/)

**Figure 2.** Each ecosystem is classified according to 10 broad habitats and defined according to eight specific modifiers. The trophic resources available for 11 trophic groups of consumers are evaluated according to three levels of abundance (1, low; 2, medium; 3, high).

The abundance of epiphytes in a shallow *Posidonia oceanica* meadow is approximately three times that recorded in a deep meadow [25]. Also, the abundance of organic detritus available for detritivore consumers is largely influenced by exposure to waves and currents [26]. Eutrophic and oligotrophic conditions influence the standing crop of primary producers, even when the same ecosystem is considered [27]. Therefore, the relative food resources estimated for each habitat (table 4a) must be tuned...
according to these site-specific influences (table 4b) and the relationship (2.1) is set as:

\[
\text{Resource abundance}_{\text{tg,ecosystem}} = f(\text{basic substratum} \times \text{specific habitat}) \times \text{specific modifiers} \tag{2.2}
\]

For this purpose, literature data were screened to detect deviations from ‘average’ expected conditions under the influence of each modifier. A value of 1 was set for each trophic category under standard conditions (table 4b), meaning that the estimate of food resources, obtained in table 4a, will not change. In contrast, exposure to modifying conditions will increase or decrease the relative amount of food resources available. For example, higher currents induce a mean decrease of 20% for the food resources available for mCa, as determined by screening the results of studies comparing similar ecosystems exposed to different strength currents [28]. Therefore, a modifying value of 0.8 was assigned in this case (table 4b).

Some modifiers produce dramatic variation from average conditions. Food resources available for mCa may be surprisingly high (330%) in oligotrophic systems [29,30], while other trophic resources (e.g. DeF and FF) are not influenced. This is reflected in the modifying value of 3.3 in table 4b, corresponding to the trophic resources mCa in oligotrophic environments.

These modifiers are applied only where documented local conditions strongly influence the relative availability of trophic resources in the considered habitats. We considered ‘shallow’ habitats those in water less than 5 m deep, and ‘deep’ habitats those located below a depth of 25 m. We considered ‘exposed’ those ecosystems open to large sea swells or characterized by very high winds, and ‘anthropogenically impacted’ those systems for which there are clear and documented evidence for major industrial, fishery or urban pressures. Thus, only a few characterizing pressures—the most evident and well documented—are considered for each site (see grey cells of table 5a), to avoid interference with the basic environmental features of ecosystems.

2.2. Application of relative available food index tables

To test the effectiveness of simulations provided by RAFIs, 19 different sites were chosen throughout the world, among those for which sufficient information was provided on the abundance of food items (permitting at least partial comparisons between computed and actual data). In fact, most studies provide incomplete sets of trophic groups and, in this case, comparisons with the whole trophic model provided by RAFI is not feasible. In particular (table 5a), each site (in rows) was classified according to its characteristics (in columns). The site descriptors (in each line) were set to ‘X’ when that specific feature was applicable, and left blank (null) when the feature was not applicable (table 5a). For example, ‘San Pietro’ (the site reported in the first row) is a eutrophic (fourth grey column), shallow (last grey column) environment in the bay of Naples (Italy), hosting a low-canopy seagrass (Cymodocea nodosa). In contrast, ‘N.E. St. Croix’ (the site reported in the 14th line) is a shallow, exposed coral community in the US Virgin Islands. Each site was similarly characterized.

This classification permitted the computation of the abundance of food items (table 5b), according to the above-described RAFIs. For example, in the case of ‘San Pietro’, the values for each trophic category were computed by multiplying all the scores previously marked with ‘X’ in table 5a, i.e. the scores in line 8 of table 4a (low-canopy seagrasses) by the scores in lines 3 and 8 (eutrophic and shallow, respectively) of table 4b, following the relationship (2). The same computation was performed for all the other considered sites (electronic supplementary material, table S2), according to their environment type and local specific pressures, as reported in the literature. Repeating this procedure, the scores for each trophic category in each site were computed (table 5b). These computations are available in digital format in electronic supplementary material, table S2, along with an empty spreadsheet to be used for the simulation of further datasets.

Finally, the values in each cell were converted, line by line, to a percentage of the total resources present in each site (RAFI%), in order to standardize the results and make them comparable among different ecosystems [31]. Thus, RAFI% (table 5c) allows comparisons among such different ecosystems as coral reefs, temperate harbours, seagrass meadows and sand bottoms, which are characterized by wide ranges of densities of organisms, dynamics and productivities.

For testing the trophic resources at three additional Australian sites (Bagot Point, Port Gawler and Barker Inlet), only the abundance of the resources for three major trophic groups (He, DeF and Ca) was reported in the literature [32]. Therefore, the per cent contributions of trophic resources for He (leaf biomass), DeF (debris biomass) and macrocarnivores (fauna greater than 1 mm), as found in the literature, were compared with the same food resources predicted by RAFI (table 6).
Table 5. (a) Classification of sites used for testing. Each site used to test the model performances is classified according to its habitat type (X, yes; white cells) and modifying conditions (X, yes; grey cells). (b) RAFI calculated for each of the above test sites (Table 5a). For example, in the case of mCa in the low-canopy seagrass San Pietro, the RAFI estimate for low-canopy seagrass (4) is multiplied by the modifiers for 'Eutrophic' (2) and 'Shallow' (1.5) to obtain a final RAFI score of 12, which is then converted to RAFF% as shown in Table 5c. The original data and computations are available in electronic supplementary material, table S2. (c) Relative abundance of food items (RAFI%) calculated for each of the test sites based on Table 5b. For example, in 'San Pietro', the trophic resources (relative abundance of food items) available for microcarnivores are 11% of the total trophic resources in this system, whereas the resources available for carnivores account only for 3% of the total resources of the ecosystem.

<table>
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<th>kelp</th>
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(Continued.)
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<th>He</th>
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<th>Om</th>
<th>mDef</th>
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</tr>
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<td>Dos Amigos</td>
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<td>2</td>
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<td>11</td>
<td>25</td>
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</tr>
<tr>
<td>Barker Inlet</td>
<td>14</td>
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<td>23</td>
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<td>23</td>
<td>4</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 6. Comparison of trophic resources reported by Edgar & Shaw [32] for three Australian sites (top part) with the results of RAFl predictions (bottom part). The proportion of trophic resources among the three main trophic groups for which experimental data were available have been calculated. Their percentages (% proportion of biomass versus RAFl%) are compared (right part of the table).

<table>
<thead>
<tr>
<th>Australian sites (as reported in the literature)</th>
<th>actual biomass (g m(^{-2}))</th>
<th>% proportion of biomass reported</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>leaf</td>
<td>debris</td>
</tr>
<tr>
<td>Bagot Point (Zostera sp.)</td>
<td>47</td>
<td>3.23</td>
</tr>
<tr>
<td>Port Gawler (Posidonia sp.)</td>
<td>206</td>
<td>332</td>
</tr>
<tr>
<td>Barker Inlet (Heterozostera sp.)</td>
<td>199</td>
<td>n.d.</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>model predictions</th>
<th>RAFl predictions (abundance units)</th>
<th>RAFl% predictions: resources for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>He</td>
<td>DeF</td>
</tr>
<tr>
<td>Bagot Point (Zostera sp.)</td>
<td>21.7</td>
<td>6.2</td>
</tr>
<tr>
<td>Port Gawler (Posidonia sp.)</td>
<td>9.9</td>
<td>24.7</td>
</tr>
<tr>
<td>Barker Inlet (Heterozostera sp.)</td>
<td>22.5</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Finally, a simulation for a marine protected area (MPA) in Africa, for which some literature information is available [33], was performed in order to test the sensitivity of the method for computing changes occurring after the institution of the protection plan. In this case, the factor ‘anthropogenic perturbations’ was set to ‘X’ before the institution and ‘null’ after the institution, to perform the simulation (electronic supplementary material, table S2).

RAFl tables were formally validated by comparing observed food resources to those predicted. For this purpose, two comprehensively sampled sites were considered: Lacco Ameno [34] and Banco di Santa Croce [35]. These sites were selected because (i) complete datasets were available and (ii) they host quite different environments (table 5a): seagrass versus hard bottom, eutrophic versus pristine, shallow versus deep, etc. Fauna was sampled using an airlift sampler [35] in two replicate 40 × 40 cm surface area plots, and all specimens collected were counted and identified at the species level.

Lacco Ameno (40°45' N, 13°53' E) is located in the northwest sector of the Island of Ischia (Bay of Naples, Italy). It contains a continuous and dense meadow of P. oceanica extending from 1 m to about 33 m (deep limit). Samples collected at a depth of 5 m were considered. Animals were grouped according to their possible role as prey for macrocarnivores, microcarnivores, filter feeders, etc. Data were integrated, when necessary, with gut content analyses evaluated for each sampled species. Prey item size was taken into account and their abundance in the environment was evaluated based on the following relationship:

\[
\text{Total food biomass available} = \text{number of items} \times \text{average individual biomass} \quad (2.3)
\]

The abundance of food available for macroherbivores and microherbivores and the actual abundance of detritus were evaluated according to [36]. The results obtained were transformed into % abundance of each food item and compared with the abundance of food items (RAFl) computed according to table 4.

Banco di Santa Croce (40°40' N, 14°26' E) is a submerged seamount complex located in the eastern Gulf of Naples. It is located 0.8 km off the coast and is composed of various rocky seamounts arising from a depth of 60 to 11 m, forming a circular structure. Samples were obtained over a 3 year extensive sampling programme to develop a trophic model for the site [37]. Direct measurements provided the actual abundance of food items and the abundance of species of each trophic group per square metre. The total number of individuals per m\(^2\), as well as the total biomass of each trophic group and abundance of organic detritus and of phyto- and zooplankton were also available [37], and converted into the same units to allow direct comparisons. The fish fauna was surveyed using visual census [37].

2.3. Statistical analyses

The \(r^2\) coefficient was calculated using correlation analysis to evaluate how well the RAFl predictions for each trophic group fitted data for the selected sites derived from the literature. The results were confirmed by the G-test (likelihood ratio test).

The actual data sampled in the two validation sites were compared with the patterns of abundance of resources obtained by means of our model, and \(t\)-tests were used to determine the significance of the difference of the slope from the null hypothesis of a 0 slope using GRAPHPAD PRISM 4 (GraphPad Software, San Diego, CA). Pearson’s product–moment correlations were also used to test agreement...
between RAFI estimated and observed food resources at the sites for which complete data across all trophic groups were available. For all the other sites, RAFI predictions were qualitatively compared with the available literature data, even when incomplete, by detecting the dominant food resources predicted by RAFI and their correspondences with the dominant food resources described in the literature.

3. Results

3.1. Relative available food index validation

The comparison of the abundances of food items estimated by means of the proposed method with field data shows some differences, but trends coincide (figure 3). In particular, data for Lacco Ameno d’Ischia (figure 3a) show good agreement between RAFI% simulated data and observed data, other than carnivores (Ca), which appear to be overestimated by RAFI. As for the other trophic categories, herbivores, DeF and DeFHe, as well as mDeF, are slightly higher when calculated by RAFI, whereas mCa, mHe, Om and DeFS are slightly lower than actual. The most abundant resource is macroherbivore food, accounting for about 25% of the total trophic resources available, followed by DeFHe (about 15%), omnivores, mHe and mCa (about 10%). On the whole, the relationship between actual and RAFI estimated data was highly significant (figure 4a, $r^2 = 0.97$).

In the case of Banco di Santa Croce (figure 3b), field data show fundamentally two types of trophic categories: those relying on low abundance resources (mCa, Ca, mHe, He, mOm, Om and FF) and those relying on locally abundant resources (mDeF, DeF, DeFS, DeFHe). RAFI predictions respect this pattern...
Figure 4. Observed values of per cent abundance for trophic resources versus RAFI% estimated values for resources present in two Mediterranean sites. The grey line denotes 1:1 agreement between the two methods. (a) Lacco Ameno; \( t = 15.72, \text{d.f.} = 9, \text{p-value} < 0.001, r^2 = 0.97 \). (b) Banco di Santa Croce; \( t = 6.38, \text{d.f.} = 9, \text{p-value} < 0.001, r^2 = 0.82 \).

\( r^2 = 0.82 \) between predicted and field data; figure 4b), apart from some variability observed in individual categories.

Similarly, t-tests indicated no significant differences (\( p < 0.001 \)) between the RAFI data simulated for three Australian sites hosting seagrass meadows and field data, according to the known feeding groups investigated (table 6 and figure 5). In addition, data reported in the literature on the abundance of the main trophic groups were compared with the results of RAFI predictions for various sites (table 7), with good coincidence.

Finally, the simulation of the Sine Saloum MPA [33] produced clear differences before and after the institution of the protection plan. In particular (figure 6), the resources available for microcarnivores, carnivores, herbivores and omnivores showed an increase in the protected conditions, whereas the trophic resources available for detritus feeders and herbivore–detritus feeders exhibited a decrease after the institution of the MPA (i.e. in the absence of ‘anthropogenic influences’).

3.2. Test of relative available food index in various sites of the world

The trophic resources available at various sites were predicted by RAFI and clear distinctions were obtained, according to specific ecological conditions, even when similar ecosystems were considered. Comparing the trophic resources available in three sites hosting seagrass meadows (San Pietro, Castello, Port Gawler), we observed very different patterns of resource distribution (figure 7). In San Pietro, which hosts a low-canopy seagrass bed (C. nodosa), most trophic resources are available for herbivores (26%), followed by detritus feeders (16%), detritus feeder–herbivores and microcarnivores (11%). In contrast, in Castello d’Ischia, an acidified site hosting a high-canopy seagrass (P. oceanica), most trophic resources are available for detritus feeders (35%), followed by DeFHe (22%) and DeFS (10%). The Australian Port Gawler site hosts a *Posidonia* sp. meadow and exhibits maximum abundance of resources for detritus feeders (25%) followed by DeFHe (16%) and DeFS (10%), showing the importance of plant detritus in this Australian seagrass ecosystem.

3.3. Relative available food index trends in various environments

RAFI computations indicated that trophic resources available for mCa reach highest abundance in several seagrass environments, coralligenous and fucoid habitats, and lowest abundance in rocky bottoms and caves. Similarly, trophic resources available for herbivores (He) reach maximum abundance in seagrass meadows and in shallow rocky bottoms, while they dramatically decrease in deep rocky bottoms and caves (table 5c). The abundance of resources available for omnivores (Om) is minimum in rocky bottoms and embayments, while detritus feeder resources (DeF) are relatively abundant in high-canopy seagrasses, caves and rocks. Finally, the abundance of resources available for FF is generally low.
Figure 5. Comparison of the results reported by Edgar & Shaw [32] on the abundance of trophic resources for He, DeF and Ca. Edgar & Shaw [32] data (E&S) are indicated by grey bars, against predictions of the RAFI model (RAFI%, white bars). Three sites are considered, for which sufficient literature data were available: (a) Bagot Point, (b) Port Gawler and (c) Barker Inlet.

and sensitive to the effect of specific modifiers, in the considered environments. In fact, according to RAFI, the abundance of food available for FF accounts for 4% of the total trophic resources in some caves (Grotta del Mago), and in an analogous environment (Formiche) it declines to 1% of the total trophic resources.

4. Discussion

4.1. The accuracy of model predictions

The availability of individual food resources in shallow marine ecosystems varies with environmental features [31], but the data published on the arrangement of resources in each ecosystem are generally
Table 7. Comparison of predicted RAFI% and abundance of trophic resources derived from the available literature. For each site, the most abundant trophic groups identified by RAFI% are indicated in the second column. The most abundant trophic group (TG) or trophic resources (TR) reported for each site in the literature (fourth column) are provided in the third column. Country abbreviations are Italy, IT; United States Virgin Islands, US; Costa Rica, CR; New Zealand, NZ.

<table>
<thead>
<tr>
<th>site</th>
<th>RAFI-predicted highest trophic resource(s)</th>
<th>most abundant trophic resources (TR) or trophic group (TG) according to the literature</th>
<th>references (electronic supplementary material, table S1)</th>
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</thead>
<tbody>
<tr>
<td>San Pietro (IT)</td>
<td>He</td>
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<tr>
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<td>DeF</td>
<td>detritivorous polychaetes (TG)</td>
<td>[132]</td>
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<td>Bell’Ommo (IT)</td>
<td>DeF, DeFS</td>
<td>gorgonians (TG)</td>
<td>[133]</td>
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<td>San Pancrazio (IT)</td>
<td>He, mHe</td>
<td>algae (TR)</td>
<td>[134]</td>
</tr>
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<td>algae (TR)</td>
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<td>algae (TR)</td>
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<td>gorgonians (TG)</td>
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<td>Grotta del Mago (IT)</td>
<td>DeF</td>
<td>detritus feeding amphipods (TG)</td>
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<td>sponges (TG)</td>
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<td>He, DeFHe</td>
<td>herbivores (TR)</td>
<td>[147]</td>
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<td>Chatham Island (NZ)</td>
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<td>detritus feeders and herbivores (TG)</td>
<td>[148]</td>
</tr>
<tr>
<td>Dos Amigos (CR)</td>
<td>Om, DeF</td>
<td>De and Om echinodermis (TG)</td>
<td>[149]</td>
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</table>

incomplete and not comparable. The proposed model represents a framework to predict the relative abundance of food resources for trophic groups present in marine areas, based on the habitat considered and local specific influences (e.g. high currents, low depth, etc.). We demonstrated that the model predictions agree well with the trophic data reported in studies undertaken in a wide range of ecosystems, both temperate and tropical.

Statistical comparisons between RAFI-predicted and observed trophic resources at two intensively studied Mediterranean sites demonstrate the accuracy of the RAFI estimates. RAFI predictions for Lacco Ameno are in close agreement with measured abundances of trophic resources. The large abundance of trophic resources potentially available for herbivores at this site was expected, since this is a *P. oceanica* environment, represented by a dense meadow exhibiting a leaf standing stock peaking at 340 g dry weight per square metre [36]. RAFI provides an appropriate estimate of the large biomass potentially available for macroherbivores. However, relatively little of this biomass is directly consumed by grazers, owing to various deterrent compounds [38,39]. Only a few herbivores, sometimes reaching high densities, are able to consume the abundant green leaf biomass, most notably sea urchins [40], some isopods [41] and a few fishes [42]. As predicted, modelled food availability does not necessarily correspond to food consumption.

The RAFI model, in fact, predicts available biomass, not consumption, and individual consumers may exploit the available resources at various levels, according to their abilities for fragmenting and
Figure 6. RAFI simulation for an MPA in Sine Saloum (Senegal), before and after the institution of the no-take area. The % abundances of trophic resources available for each feeding category are reported. The area is composed of a ‘sand’ bottom and contains some ‘natural perturbations’. Therefore, these two indicators were set to ‘X’ in the classification of the sites. In addition, to simulate the local food webs before the MPA institution, the indicator ‘anthropogenic perturbations’ was set to ‘X’; to simulate the local food webs after the institution of the MPA, the indicator ‘anthropogenic perturbations’ was shifted to ‘nil’. The corresponding scores (table 4a,b) were multiplied according to the relationship (2.2).

detoxifying food items [43]. Consequently, the abundance of resources estimated by RAFI represents the potential abundance of food accessible for each category of consumers, and is independent of the ability of individual species to exploit the resource (top-down control).

The second most abundant food resource in Lacco Ameno, based on both RAFI and observed data, is for DeFHe. Fundamentally, this is plant detritus, which is very abundant in P. oceanica meadows [44,45] and, in particular, in Lacco Ameno, where 42% of the plant primary production is transformed into detritus that is degraded in situ [36]. This large biomass is available for several consumers, including crustaceans and some echinoderms [46].

A divergence between RAFI and observed data at Lacco Ameno was found for macrocarnivores (Ca). However, ‘macrocarnivores’ in P. oceanica ecosystems are principally represented by fishes [47], which often consume other fishes [48,49], whereas invertebrate macrocarnivores are present only in the rhizome layer and they are represented by a few species of large decapods and echinoderms [50]. Interestingly, literature data on fish stocks could not be considered for the evaluation of the actual biomass, since the methods applied for their collection in this site did not refer to a surface area [51]. In contrast, the abundance of other trophic resources was evaluated on a surface unit base, according to the literature [17,34,36]. If the fish fauna was considered and added to the actual abundance of resources for carnivores, this value would increase substantially. Thus, RAFI arguably provides a more reliable value for the abundance of carnivore trophic resources than data obtained from the literature, because the abundance of fish per surface unit was not precisely evaluated through field investigations.

This outcome emphasizes the need for development of a general model to estimate trophic resources. RAFI estimates trophic resources of ecosystems while avoiding methodological constraints hindering comparison of food resources measured with different scales or units. In fact, owing to methodological constraints, researchers generally consider only a subset of trophic resources, which can lead to incorrect conclusions when different environments are compared.

4.2. Further validation on a rocky environment

The RAFI estimates for Banco di Santa Croce indicate two distinct categories of trophic resources: those present in low abundance (less than 5% of total trophic resources), such as those sustaining populations of carnivores, herbivores and omnivores, and those present in large abundance, all linked to the organic detritus. Food webs in this rocky area are mostly established on the organic detritus deriving from Sarno River [52,53] and a good statistical match between actual data and RAFI estimates was demonstrated.

The largest difference between RAFI-predicted and observed trophic resources at Banco di Santa Croce was in the resources available for FFs. However, this particular site is characterized by an exceptional biodiversity and abundance of FFs (sponges, gorgonians, corals, etc.), which together must
rapidly deplete available trophic resources [37]. Therefore, the abundance of food for FFs, as sampled, is potentially low, owing to rapid consumption by animals according to a very strong top-down control of their abundance. In this case, the RAFI value, indicating the abundance of resources potentially available for these organisms, could be closer to an index of production.

Throughout this study, we have considered food abundance as a proxy for production because very few studies describe production for a range of food items. Nevertheless, at locations with rapid turnover of particular dietary items this assumption may introduce over-prediction, compared with measured values [5] of standing stocks. The actual abundance of trophic resources measured in the field (i.e. their standing stocks) is determined by bottom-up control (the amount of production) as well as top-down

Figure 7. Distribution of trophic resources (expressed as RAFI%) for three selected sites containing seagrasses: (a) San Pietro, Cymodocea nodosa meadow in the Bay of Naples; (b) Castello, Posidonia oceanica meadow established in a highly acidified area off the island of Ischia (Italy); (c) Port Gawler, Posidonia sp. meadow in Australia.
control, owing to the activity of consumers. Therefore, measured divergences from the RAFI model of resource distribution might be used to improve our understanding of real ecosystems, the effects of human disturbances, the propensity of ecosystems to be invaded and their overall stability as a result of boom and bust dynamics at given trophic levels.

4.3. Relative available food index tested at sites in the world

The RAFI tables computed in this study demonstrated good predictions of the relative trophic resources available to each trophic group in the ecosystems tested, coinciding with the most abundant trophic resources, or the trophic groups feeding on them, at several coastal sites worldwide (table 7). Also, the sensitivity exhibited in the simulation of the MPA in Senegal (Sine Saloum Delta) is remarkable. In fact, a specific investigation [33] found, as a consequence of the MPA institution, a decrease in the abundance of herbivore–detritivore fish (from 44.0% to 6.3% in biomass), and a decrease in FF–microplanktivore fish (from 31% to 12.5% in biomass) when compared with a significant increase of carnivore and omnivore fish (from 5.9% to 49.6% and from 5.2% to 11.8%, respectively). These data are in accordance with the scenarios provided by RAFI, indicating a clear decrease of trophic resources for DeF and FF, and an increase of resources available for Ca and Om, although our computations pertain to the whole food webs (including all animal taxa) of the area, whereas the data available in the literature are referred to the fish compartment only. The total biomass of consumers is related to the abundance of their trophic resources [6]. Therefore, a general agreement between the estimated available resources and the actual abundance of their consumers was found, although published data are insufficient for formal comparisons.

RAFI tables require further tests to extend the general applicability of the proposed model to other ecosystems [31]. Nevertheless, the RAFI framework developed to describe the trophic resources available in specific habitats and the modifying effect of local conditions can now be applied and tested in any natural ecosystem worldwide.

Ethics. Collections of animals were performed on board the vessel Phoenicia of the Stazione Zoologica Anton Dohrn, according to the permissions to collect organisms for scientific research granted to the Stazione Zoologica by ‘Capitaneria di Porto’ and ‘Ente Area Protetta Regno di Nettuno’. No special ‘Animal Care Protocol’ was required according to the law.

Data accessibility. All data and supplementary material are available at the Stazione Zoologica Anton Dohrn and in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.nt91j [54].

Authors’ contribution. All authors were involved with the data analyses and the writing of the manuscript. V.Z. designed the study, collected the literature information, coordinated the study and drafted the model. V.Z. and T.J.A. analysed the database, performed statistical analyses and improved the model according to the suggestions of reviewers. G.J.E., V.Z. and T.J.A. interpreted the results, discussed the methods and wrote and critically revised the manuscript. All authors gave their final approval for publication.

Competing interests. The authors declare no competing interests in the fields covered by this study.

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References

Trophic cascades: abiding

There are real differences among

and ecological parameters

Spatio-temporal

heterogeneity and the trophic level concept at the

system: the role of habitat type and food

variability in

Global sensitivity analysis of an end-to-end marine

cascades in the regulation of plankton communities

Combined effects on zoobenthic community, populations and

and the loss of phenolic substances in

...
Section 4. General Discussion
4.1. Food webs: an historical perspective

The investigations reported in the Section 2 indicate the advantages and the constraints of various methods of investigation of food webs. It is interesting to observe that even simple numerical analyses of gut contents data (Cpt. 1) indicate a disproportion in the diversity of consumers feeding on different layers of a seagrass meadow. In particular, the relatively small biomass of epiphytic algae sustains a large variety of (small) consumers, including polychaetes, crustaceans, molluscs, echinoderms and fish. In contrast, the large biomass stored into the leaves of the seagrass itself is consumed only by a few species of (large and numerically abundant) echinoderms and fish. The classification of species in trophic guilds (Cpt. 2) permits a better definition of various categories of consumers exploiting the above-mentioned resources and fundamentally confirms this general picture. In addition, the data obtained on trophic guilds will be critical to develop the RAFI index (Cpt. 6), as a further step to reach the final result of this thesis, i.e., finding an equation linking biodiversity and resource availability.

A primary driver of patterns of biodiversity is the heterogeneity in the amount of energy available, or the primary productivity measured at a given location (Lotka, 1956; Mittelbach et al., 2001). Biodiversity itself is heterogeneously distributed across the Earth (Gaston 2000) and ecological theory (Oksanen & Oksanen, 2000) suggests that food webs are structured by the interaction of resources (Chase, 2000) and their consumers (Leibold, 1977; Persson, 1999; Polis, 1999). For example, the abundance of herbivores and their predators increase along a gradient of primary productivity (Chase, 2000). Although the shape of the relationships between productivity and species diversity is variable (Waide et al., 1999; Gross et al., 2000), the abundance and availability of food
may influence the patterns of biodiversity in natural systems (Sokolowski et al., 2012).

For this reason, studies reported in Cpt. 3 are fundamental to confirm the actual productivity of a seagrass itself (*Posidonia oceanica*) and of its epiphytes, along with the factors modulating the seasonal patterns of production because this helps understanding also the seasonally variable trends of production as compared to the biodiversity exhibited by various trophic compartments. Studies on plant communities suggest that species diversity is correlated to productivity and that the hump-shaped productivity-diversity relationship is correlated to the amount of nutrients in the soil (Tilman and Pacala, 1993). These investigations demonstrate that “diversity is a unimodal function of productivity and other measures of nutrient supply rates”. Similarly, ecological models predict that species diversity is correlated to productivity as well as to the abundance of trophic resources, and that the productivity-diversity relationship should be unimodal (Tilman, 1993; Rosevzweig, 1995).

Part of these relationships may be explained in the light of the information contained in various food items, since the biomass of the seagrass may be considered “chemically protected” in respect to that of epiphytic algae and the latter may contain information able to influence the life cycles of consumers (Chapters 4, 5).

Finally, we know that body size is an important factor in the competition for natural resources (Bagchi and Ritchie, 2012) and that a smaller consumer should be competitively superior across a wide range of supplies of the two resource types (Persson, 1985). Productivity, trophic resource availability and body size must thus be interrelated in natural systems (Schoener, 1976; Schroeder et al., 2009); however previous research has been focused mainly on the influences of predators on the populations of prey, through a top-down approach (Polis and Strong,
1996; Polis, 1999; Pawar et al., 2012; Braga et al, 2012). Here we wish to explore the relationships between species diversity and the abundance of trophic resources at various sites around the world, taking into account a variety of environments (hard and soft bottoms, seagrass meadows, caves, etc.), along a range of anthropic impacts, to test the hypotheses above reported, i.e., that:

1) a relationship exists between abundance of trophic resources and biodiversity;

2) that this relationship has the shape of exponential decay, as indicated by network theory;

3) that in case of exponential decay the exponent is close to 1/3 due to allometric scaling and

4) that the higher availability of trophic resources (evaluated according to Chapter 6) coupled to lower $S$, must be accompanied by higher number of individuals or higher individual biomass (or both) to provide full exploitation of available resources.

Evidences for dependence of biodiversity patterns on the local partitioning of food resources will be also presented, in order to clarify the relationships between functional biodiversity and allocation of resources, according to a theoretical relationship derived from mathematical treatment of network data, represented by the scaled numbers of resources and consumers (as reported in Section 1), and roughly represented by a function of exponential decay (derived from the equation (24):

$$Diversity \ of \ consumers = K \ast \ exp(\ -R)$$

Where $K$ is a variable depending on the life cycle and turnover of consumers according to their feeding guilds and $R$ could be roughly...
evaluated to be close to 1/3. If all the above will be demonstrated true, we should also hypothesize, as indicated in previous chapters, that the larger abundances of trophic resources, eventually used by a low diversity of consumers (scaled numbers) will correspond to an increase in numerical abundance of consumers, or an increase of their body size, to be efficiently exploited (if not physically or chemically protected) according to a linear relationship in the shape of:

\[
Nr.\text{ consumers} \times \text{Size consumers} = r \times \text{trophic resource biomass} \quad (27)
\]

Also in this case “\(r\)” is a variable depending on the turnover and the life cycles of consumers. The above-mentioned two relationships have been tested over actual field data to validate our hypotheses. In conclusion, we tried to answer the questions:

i) Is biodiversity influenced by the abundance of trophic resources in different marine systems?

ii) Can we predict the level of functional redundancy in a given system taking into account the relative abundance of the main prey for each trophic group?

iii) Does compartmentalization and diversification of trophic resources within a system allow for a spread of species diversity?

To test these hypotheses, relationships between theoretical food availability and the species diversity according to feeding guilds were compared. In particular, to check the possibility for a wide application of the relationships above reported, the following experimental data were taken into account:

- extensively (to test the above mentioned hypotheses): 13 stations distributed into the bay of Naples and 41 world sites comprised in a large
coral fish data-base, for which large datasets of biodiversity were available from direct measurements and from literature;

- **Intensively** *(to validate the model obtained)*: two Mediterranean sites for which detailed data on size of individuals and actual abundance of food items were collected by means of different techniques.

### 4.2. The RAFI% index

One of the main constraints to compare trophic resources across different environments is the lack of equivalent information on their actual abundance. For this reason, we developed a procedure to evaluate the trophic resources theoretically available for each trophic group in any marine ecosystem. Using specific indices (Zupo et al., 2017; Chapter 6 of this thesis), we obtained final scores indicating the Relative Abundance of Food Items (RAFI%) for each of the considered environments. The same scores are applicable to any marine environment, by classifying it according to the ecosystem (hard bottoms, soft bottoms, seagrasses, kelps, etc.), and local site deviations from the conditions of “standard” inshore ecosystems (e.g. exposed, eutrophic, deep, shallow, etc.). Consequently, a score was obtained for each trophic group and for each ecosystem and it indicates the theoretical abundance of a given food item (not the actual rate of ingestion by its consumers). In fact, a seagrass meadow may contain, for example, a huge abundance of plant biomass, but this is not directly consumed in the majority of cases, being chemically protected. As well, a rocky bottom may contain a large abundance of food items available for carnivores, but they may be not consumed due to the lack of larger fish in the specific area (Zupo and Stübing, 2010). For this reason, the RAFI scores are representative of the “theoretical” food availability, not of the real consumption by various feeders.
### 4.3. The shape of biodiversity

Species diversity had a statistically significant, inverse exponential relationship with food abundance (Fig. 12a), consistently in all Mediterranean sites. Similar relationships were found when individual environments are investigated (Fig. 12b and 12c). The highest diversity corresponds to species feeding on “less abundant” food items and vice-versa. In contrast, average size of species within a trophic group correlated with the abundance of their food items ($R^2= 0.73; p<0.01$). While this last result appears obvious and in accordance with specific literature (McClain and Boyer, 2012; McClain et al., 2012), because an increased availability of food positively influences individual biomass and total abundance of consumers in natural populations, the previous results on the inverse relationships between diversity and food availability may look in contrast with instinctive assumptions (Sokolowski et al., 2012). Since species diversity is positively related to productivity (Mittelback et al., 2001; Gross et al., 2000), one would expect a similar pattern in the local abundance of food resources. However, as demonstrated in the previous chapters, this evidence confirms the results of the modelistic approach.

Several instances, at least in benthic systems, confirm this trend. For example, two main sources of primary production are available in a *Posidonia oceanica* meadow (Mazzella and Zupo, 1995): the leaf tissues and the epiphytes living on the leaf stratum. Leaf growth is slow, while the productivity of epiphytes is high (Gacia et al., 2009). The standing crops of these two main food sources per unit area are very different, as the biomass of *Posidonia* leaves exceeds by three orders of magnitude that of epiphytes (Terrados and Medina-Pons, 2011), but studies on the diversity of species classified into trophic groups, and feeding on the leaves of the
seagrass or on its epiphytes, demonstrated that only a few grazers feed directly on seagrass tissues (Vizzini, 2009), while a complex and diverse grazer community feeds on the “scarcely abundant” epiphytes (Gacia et al., 2009). Such evidence, which could easily be replicated for hard-bottom environments as well as for sand-bottoms and caves, explains our results about the inverse relationship of biodiversity with trophic resources. Highly eutrophic lakes provide a further non-marine example, as these are characterized by low diversity of species, large abundance of organic matter available for feeders and high productivity (Mukherjee et al., 2010).

These results, on the whole, may be explained in the light of the arguments raised by Bagchi and Ritchie (2012). They developed equations able to provide predictions on the “diversity” of diet reaching large size or small species in the same environment and, interestingly, the graphical representation of their equations predicts that smaller consumers are ordered in the left-top part of the diagram and are characterized by a higher dietary diversity, as compared to larger consumers, whose diet is dominated by a single resource (right, down part of the graph; Figure 12d). This theoretical diagram fits both the theoretical model and our experimental findings. Thus, the constant trends of biodiversity we found may be viewed in the light of this set of body size-based allometric constraints (Bagchi and Ritchie, 2012).
Figure 12. (a) Relationship between % alpha diversity versus % RAFI for trophic groups at 13 Italian sites. Dashed line represents 95th log-linear regression quantile (Cade et al., 2003). R² for 95th regression quantile = 0.373 (p<0.001). (b) Food availability versus diversity in trophic groups at comprehensively surveyed seagrass meadow Lacco Ameno. Species list accumulated over multiple surveys. Abundance of food items calculated on actual data of numerical abundance of food items per unit area and results of gut content analyses. Multiple R-squared: 0.8521 (p<0.001). (c) Relationship between alpha diversity and food availability for hard-bottom site Banco Santa Croce. Food resources were produced using Ecopath-Ecosim. Each point represents a trophic group (n = 5) at a sampling station (n = 4) for a seasonal survey (n = 4). Multiple R-squared: 0.3477 (p<0.005). (d) Mathematical predictions about the distribution of trophic resources between small and large consumers are perfectly superimposable to the experimental results. Most data points (Figures 12 a, b, c) for small consumers are ordered in left area of this figure, while a few larger species consume the most abundant resources, and they are ordered in the lower area (From Bagchi and Ritchie, 2012).
4.4. Experimental validation of the model

Alpha diversity at Lacco Ameno (Island of Ischia, Italy), a continuous seagrass meadow, is significantly correlated to the abundance of food items ($R^2= 0.81; p<0.01$). The analysis in this case was based on observed data on the numerical abundance of food items per unit area and results of the gut content analyses. Taxonomical analyses indicated that filter feeders and micro-omnivores were the most diverse groups in the system (120 and 63 species, respectively) and they fed on the lowest abundant trophic resources (number of prey equals 1.04 m$^{-2}$ and 2.08 m$^{-2}$, respectively). In contrast, detritus feeders, herbivores and detritus feeder-herbivores were the least diverse feeding groups (3, 4 and 5 species, respectively) and they fed on the most abundant trophic resources (number of prey equals 21.0 m$^{-2}$, 21.0 m$^{-2}$ and 17 m$^{-2}$, respectively). Size of individuals and number of individuals per square meter, as expected, were directly correlated to the abundance of food items ($R^2= 0.63$ and 0.58, respectively). In fact, filter feeders and micro-omnivores had the lowest average size (2.7 and 2.9 mm respectively), and the lowest number of individuals (0.2 and 1.4 ind./m$^2$, respectively), while detritus feeders, herbivores and detritus feeder-herbivores had higher average size (6.5, 7.3 and 4.9 mm, respectively) and higher number of individuals (4.8, 6.2 and 3.9 ind./m$^2$, respectively). A similar picture was obtained at Banco di Santa Croce. In this case, Alpha diversity evaluated on a benthic hard-bottom system demonstrated a trend with the abundance of food items that is ($R^2=0.35$; Figure 12c) consistently described by an exponential equation.

The use of actual data on the abundance of food items (kJ m$^{-2}$, according to the Ecopath model) represents an experimental confirmation of the results obtained by means of the extensive study at Banco di Santa Croce, but original data do not allow the fine definition of macro-consumers and
micro-consumers, as needed for applying the RAFl index (Zupo et al., 2017). However, the inverse relationship indicates that higher diversity is correlated to lower abundance of food items, obtained in this case by direct measures. The most diverse groups in this specific hard bottom environment are herbivores (85.7 species, on average, in the 4 stations) and carnivores (79.7 species, on average), and they fed on minor (less abundant) food items for this environment: the abundance of food items in this case reaches average values (in 4 sampling stations) of 51.2 kJ m\(^2\) for herbivores and 15.5 kJ m\(^2\) for carnivores. In contrast, filter feeders and detritus feeders are the least diverse groups (24.7 and 40.5 species, on average, into the 4 stations representing the site “Banco”) and they fed on the most abundant food items: the abundance of food items reached average values of 55.6 kJ m\(^2\) and 70.4 kJ m\(^2\), respectively. These data confirm previous findings indicating that biodiversity patterns are in accordance with the trends of environmental energy availability (Gaston, 2000).
Figure 13. (a) Biomass of trophic groups linearly increased with food availability on a log-log scale. Food resources were produced using Ecopath-Ecosim. Each point represents a trophic group (n = 5) at a sampling station (n = 4) for a seasonal survey (n = 4). Multiple R-squared: 0.7657 (p<0.001). (b) Number of individuals in trophic groups linearly increased with food availability on a log-log scale. Food resources were produced using Ecopath-Ecosim. Each point represents a trophic group (n = 5) at a sampling station (n = 4) for a seasonal survey (n = 4). Multiple R-squared: 0.4424 (p<0.001)
Total biomass (expressed as kJ m\(^{-2}\)) and number of individuals per square meter are, as well, directly correlated to the abundance of food items (Figures 13a and 13b): herbivores and carnivores have lower biomass, on average (340.7 and 126.5 kJ m\(^{-2}\) respectively), and lower number of individuals (456.5 and 187.9 ind./m\(^{2}\), respectively), while filter feeders and detritus feeders have the highest biomass (3336.7 and 699.3 respectively) and higher number of individuals (226.6 and 229.7 ind./m\(^{2}\), respectively).

**The RAFI table**

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<th>mDef</th>
<th>Def</th>
<th>DeFS</th>
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**Site specific features**

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**Table 1.** Computation of RAFI indices for various environments, according to trophic categories. (a). RAFI (relative food availability) developed in Zupo et al., 2016 as a tool to analyse any coastal ecosystem. (b). Table of site specific features to be assigned to individual environments, to further tune the theoretical data contained in table A (after Zupo et al., 2016). To calculate the theoretical abundance of food items in a given environment the user identifies in table A the substrate corresponding to specific sites (e.g., high canopy seagrass) and in table B one or more specific features, if applicable. The corresponding scores must be multiplied among them to obtain the RAFI value of each food item.
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Table 2 (a). Site specific features. Each site is indicated by its main characteristics, as reported in tables 1A and 1B. The 13 Mediterranean sites (on the left) and 8 of the 41 RLS sites are shown.
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**Table 2 (b).** RAFT (relative abundance of food items) calculated for each of the above sites, according to the previous tables.
The application of the RAFI% scores (Table 1a and b) to 41 ecosystems described in the global diversity data-base confirmed the inverse relationship Alpha versus RAFI% (Figure 14), as well as the significant direct relationships RAFI% versus size and RAFI% versus numerical abundance, so demonstrating its global applicability. Hence, the peculiar relationships detected on 13 Mediterranean sites and validated by intensively studying two very different benthic ecosystems (a seagrass meadow and a rocky bank, as above described) have been confirmed on 41 different environments (from sandy bottoms to coral reefs) located in a wide portion of the globe. This process of development, validation and confirm demonstrates the possibility to use the RAFI% tables (Zupo et al., 2017) as a general tool applicable to any environment, to provide comparisons of the general trends of biodiversity as related to the abundance of food items locally present.

**Figure 14.** Limiting influence of increasing food availability on alpha diversity of trophic groups for 41 sites representing shallow benthic habitats around the world. $R^2$ for 95th log-linear regression quantile = 0.158 ($p<0.001$).
Section 5. General Conclusions
5.1. The equations of life

In conclusion, biodiversity is inversely correlated to the abundance of food items according to the exponential relation (26), while the size of individuals and their numerical abundance are linearly correlated to the levels of food availability according to the relation (27), confirming the findings of previous investigations (McClain & Boyer, 2012; McClain et al., 2012). Two groups of species appear to be consistently recognizable in any marine ecosystem (Fig. 15), according to their trophic features: a first group, feeding on “abundant” food items, characterized by low taxonomical diversity, large abundance and, generally, large size, and a second group, feeding on less abundant food items, characterized by high taxonomical diversity, low abundance and, generally, small size. The presence of the two groups may be understood as a result of variable defence abilities by the preys (Speed and Ruxton, 2007), since some species may invest energy in the production of structural or inducible defences, and they drastically reduce the diversity of their consumers, while other species may invest most energy in the production of new biomass (turnover) and in this case their crop is totally available for a large diversity of consumers (Stanley, 2007). Following this pattern, the consumers able to feed on “defended” species have only a few competitors and they can thus rely on a large abundance of trophic resources: their populations reach large biomass and numerical abundance. In contrast, the consumers of “secondary” items, exhibiting a higher turnover and a lower level of defence, may have several competitors (high alpha diversity in the same trophic group) that limit the potential expansion (numerical and in terms of biomass) of their populations. In fact, according to Stanley (2007), “the basic idea that competition can set a limit for marine animal diversity is incompatible with
In addition, Basset and Angelis (2007) suggested that smaller consumers can counter the competitive advantage of a larger competitor by their ability to reduce resource densities to a low level and, in this view, a higher diversity of smaller consumers could be forecasted for less abundant resources, in any ecosystem, as demonstrated by our findings, although previous studies have never explicitly connected body size to mechanisms of competitive coexistence of the consumers. According to the assumption of our theoretical model, however, we do know that the widely documented patterns of size-related niche partitioning may be due to relationships of competitive coexistence (Figure 15).

**Figure 15.** Graphical sketch of the main results of this study. Two groups of species are consistently found in all ecosystems, the first represented by cryptic species of small size, characterized by high diversity, the second represented by larger species, characterized by low diversity.

These conclusions open the possibility for testing various hypotheses in selected ecosystems. For example, we demonstrated that biodiversity is inversely correlated, in each trophic group, to the maximum size reached
by species (Figure 16a). Since global patterns of biodiversity indicate an increase of species diversity from higher latitudes towards the lower ones (Gaston, 2000), can we accordingly forecast a decrease of size moving from poles to the equator? An analysis of the global biodiversity database confirms this hypothesis and shows that also this trend is confirmed. As well, taking into account single trophic groups, since our model forecasts a decrease of diversity according to the availability of trophic resources, can we demonstrate a decreasing trend for the species diversity in the group of herbivores, in various environments, according to the percent cover of various plants? Also in this case the Global Biodiversity data-base allowed to confirm this hypothesis (Figure 16b).
Other hypotheses could be formulated, about the role of small sized organisms (Figure 15) present in a given environment (feeding on secondary items?), the exceptional biodiversity recorded in some areas (importance of secondary prey?), or the quick disappearing of large size species according to dramatic modification of the food webs (disappearance of “structural” food items?).

Even more interestingly, considering once again that our model of biodiversity was based on mathematical properties of networks, the trends observed of biodiversity and abundance of trophic resources should be transferable to any other biological system in which a network of cells or organisms is connected by trophic relationships. In this view,
we could forecast that, in agriculture, the addition of fertilizers will trigger larger production of a few crops, while the scarcity of resources will facilitate the increase of biodiversity, with the introduction of several (generally undesirable) species. Similarly, we can forecast that in a single organism, an abundance of trophic resources for cells (e.g., plenty of sugars, aminoacids and fatty acids) will facilitate the production of a single type of cells (eventually pathological?), while the “biodiversity” in the tissue homeostasis could be promoted by the scarcity of trophic resources.

The aforementioned hypotheses and several others will be tested in further investigations, in order to establish the actual limits of the model herein developed, linking the biodiversity levels to the availability of trophic resources and based on the above stated function of exponential decay. This will also facilitate a general interpretation of biodiversity patterns observed in any ecosystem, and a thorough comparison of their trophic structures. Since the equations here tested were derived from a mathematical evaluation of networks and further tested on ecosystem data, it is likely that they can find confirmation and validity also in other networks of live structures (cells, tissues, species, ecosystems) at various organization levels. Further studies will explore this hypothesis and eventually extend the validity of the two equations to a range of biological systems.
Section 6. References


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**Figure 13.** (a) Biomass of trophic groups linearly increased with food availability on a log-log scale. Food resources were produced using Ecopath-Ecosim. Each point represents a trophic group \((n = 5)\) at a sampling station \((n = 4)\) for a seasonal survey \((n = 4)\). Multiple R-squared: 0.7657 \((p<0.001)\). (b) Number of individuals in trophic groups linearly increased with food availability on a log-log scale. Food resources were produced using Ecopath-Ecosim. Each point represents a trophic group \((n = 5)\) at a sampling station \((n = 4)\) for a seasonal survey \((n = 4)\). Multiple R-squared: 0.4424 \((p<0.001)\)

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**Figure 15.** Graphical sketch of the main results of this study. Two groups of species are consistently found in all ecosystems, the first represented by cryptic species of small size, characterized by high diversity, the second represented by larger species, characterized by low diversity.

**Figure 16.** (a) World trends of size for fish and invertebrates, along a latitudinal scale, show that the predictions of our trophic model (higher biodiversity is related to lower size) are confirmed. (b) RLS sites. Relationship between algae percent cover and richness of fish species calculated using the Global Biodiversity data-base. Dashed line = 90th regression quantile. \(R^2\) for 95th log-linear regression quantile = 0.120 \((p<0.001)\)