Microbial and Metabolic Dynamics in Sponges under Ocean Acidification

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Microbial and Metabolic Dynamics in Sponges under Ocean Acidification

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ABSTRACT

Ocean acidification (OA) poses a significant threat to marine ecosystems, and benthic organisms must develop adaptive strategies. Despite being frequently regarded as ‘winner taxa’, we lack a comprehensive understanding of how sponges tolerate stress and undergo positive acclimatization. Marine sponges can be categorized as high or low microbial abundance (HMA or LMA) species, which may adopt distinct strategies to maintain homeostasis and fitness under changing conditions. This study investigated adaptive traits of the HMA sponge *Chondrosia reniformis* and the LMA sponge *Spirastrella cunctatrix* by comparing microbial and metabolic shifts in sponge holobionts collected from a natural CO$_2$ vent system and a control pH site in Ischia, Italy. Microbial diversity and core microbiomes changed for both species in response to OA. Morphologically, *S. cunctatrix* exhibited tissue necrosis accompanied by reduced oscula and water canal sizes, indicating a stress-induced dysbiosis and microbial instability. In contrast, *C. reniformis* appeared to benefit from a highly diverse microbiome with functional redundancy and local microbiome stability, promoting acclimatization to OA. NMR-based metabolomics revealed stable metabolite profiles across sites for *C. reniformis*, indicating metabolic homeostasis, whereas metabolic shifts in *S. cunctatrix* suggested OA interference in several pathways, including osmoregulation and energy metabolism.

To expand on organismal acclimatization processes towards biochemical exchanges with the environment, a new *in situ* sampling methodology was developed. The study of inhaled and exhaled water fluxes of filter-feeders was improved using a submarine peristaltic pump, which was tested on *C. reniformis*. The new methodology can be applied to various ecological research topics, such as nutrient cycling, filter-feeding fluxes, plankton dynamics, and seawater metabolomics. This dissertation compares diverse OA acclimatization strategies of two co-occurring Porifera species in a CO$_2$ vent system based on microbiome and metabolic patterns. Moreover, parallel studies of biochemical exchanges with seawater are crucial to reconstruct these adaptation mechanisms.

Keywords: Ocean Acidification, Microbiome, Metabolomics, Porifera, Climate Change, CO$_2$ vents, InEx filter feeding fluxes
**Graphical Abstract**

![Graphical Abstract Image]

**Figure 1:** Microbial and metabolic responses in HMA and LMA sponges in response to ocean acidification. The high-microbial abundance (HMA) sponge *Chondrosia reniformis* shows different microbiome and metabolome changes compared to the low-microbial abundance (LMA) counterpart *Spirastrella cunctatrix*. Complex microbial and metabolic responses are correlated to a healthy physiology of the HMA sponge lacking morphological changes. The LMA sponge exhibits smaller oscula, aqueous canals and a reduced body surface, indicating physiological stress.

**Figure 2:** PeriSIP - a new developed methodology to sample inhaled and exhaled water fluxes from filter feeders. In complex reef systems, sponges, corals and algae release dissolved organic matter (DOM) into the water column which are, along with ultra-nanoplanktonic cells and heterotrophic bacteria, inhaled by sponges and metabolized. Exhaled seawater composition is changed and shows a high retention of different trophic cell types (e.g. *Synechococcus*, picoeukaryotes, bacteria), while fertilizing the environment through nutrient recycling processes. PeriSIP sampled seawater allows numerous downstream analyses, including the study of (in)organic nutrients, nano-planktonic cells, microbiome, metabolites, and dissolved gases.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ASV</td>
<td>Amplicon sequence variant</td>
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<tr>
<td>BCAA</td>
<td>Branched-chain amino acids</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>Chloroform</td>
</tr>
<tr>
<td>DOM</td>
<td>Dissolved Organic Matter</td>
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<tr>
<td>Ex</td>
<td>Exhaled</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell counting</td>
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<td>FID</td>
<td>Free induction decay</td>
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<tr>
<td>HGT</td>
<td>Horizontal gene transfer</td>
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<tr>
<td>HMA</td>
<td>High microbial abundance</td>
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<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum coherence spectroscopy</td>
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<tr>
<td>In</td>
<td>Inhaled</td>
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<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
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<tr>
<td>LMA</td>
<td>Low microbial abundance</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MPI</td>
<td>Max Planck Institute</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OA</td>
<td>Ocean acidification</td>
</tr>
<tr>
<td>OPLS-DA</td>
<td>Orthogonal projections to latent structures discriminant analysis</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analyses</td>
</tr>
<tr>
<td>POM</td>
<td>Particulate organic matter</td>
</tr>
<tr>
<td>SZN</td>
<td>Stazione Zoologica Anton Dohrn of Naples</td>
</tr>
<tr>
<td>TOCSY</td>
<td>Total Correlation Spectroscopy</td>
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“Symbiosis is a much higher reflection of intelligent life.”

— Frederick Lenz
CHAPTER I

GENERAL INTRODUCTION
Ocean acidification (OA) is a global environmental cataclysm, which has developed since industrialization as a result of increased atmospheric carbon dioxide concentrations. Seawater is partially absorbing CO₂ and mean oceanic pH has already dropped from 8.2 to 8.1 over the last century, corresponding to an increased acidity of about 30% (Caldeira and Wickett, 2003; Doney et al., 2009; United Nations, 2015). Average seawater pH values though, have been relatively stable over the last million years. In fact, during the last cold glacial period seawater pH averaged around 8.3 over 10,000 years, and pH changes progressed at slow rates close to 0.0003 pH units per decade. Then, over the last 200 years, anthropogenic activities rapidly increased CO₂ emissions, dramatically accelerating this process at rates close to 0.015 pH units per decade (Haugan and Drange, 1996; Hönsch and Hemming, 2005). Future outcomes are concerning, predicting a fast destabilization of our oceans.

OA has been assumed as an environmental threat by the United Nations, and its stabilization is addressed as one goal of their Agenda for 2030, in concomitance with a common global sustainable development over the next decade (United Nations, 2015). Framework Convention on Climate Change in 1992 (UNFCCC) already formulated to stabilize greenhouse gas emissions into the atmosphere, ‘at a level that would prevent dangerous anthropogenic interference with the climate system [...] within a time-frame sufficient to allow ecosystems to adapt naturally to climate change, to ensure that food production is not threatened, and to enable economic development to proceed in a sustainable manner’ (United Nations, 1992). However, international climate negotiations have only invested minimal consideration into ocean impacts, especially those related to ocean acidification (Harrould-Kolieb and Herr, 2012).

Between 1970 and 2002, global emission of CO₂ increased by over 70% (Alhorr et al., 2014). Anthropogenic CO₂ emission occurs from quotidian activities including: deforestation, land clearing for agriculture, soil degradation, cement production, and from burning fossil fuels (e.g., coal, natural gas and oils) for power generation, road and air travel (Azevedo et al., 2018; Freund, 2013; Yoro and Daramola, 2020). During all these activities, CO₂ is released into the atmosphere. On the other end, intensive land use removes natural CO₂ sinks, such as forests, in which plants absorb CO₂ as part of the carbon life cycle and naturally remove (or sequester) it to produce sugars and oxygen (Figure 1.1). The balance of all these processes results in a significant increase of greenhouse gas emissions to our atmosphere (Amaral et al., 2019). Oceans act as major sinks of CO₂, and as atmospheric CO₂ dissolves in their surface, if forms carbonic acid and free H⁺, increasing ocean acidity. While the concentrations of inorganic carbon species and CO₂ increase, carbonate ions (CO₃²⁻) decrease, changing the biochemistry dynamics of the seawater system (Caldeira and Wickett, 2003; Doney et al., 2009; Gattuso and Hansson, 2011). As a result, calcification becomes more difficult in an increasingly acidified environment, because carbonate concentrations
decrease, a key structural component for carbonic shell formation, while more bicarbonate and carbonic acid is produced (Andersson and Gledhill, 2013; Doney et al., 2009). Marine organisms which depend on calcium carbonate as key component in the formation of calcareous skeletons and shells are particularly sensitive to OA (Fabry, 2008; Kroeker et al., 2010). This was confirmed by a great number of research publications, investigating negative impacts on calcifiers and their biodiversity (e.g. Andersson and Gledhill, 2012; Chan and Connolly, 2013).

Figure 1.1: Ocean Acidification. CO₂ emissions by human activities such as the burning of fossil fuels, cement industry and transportation. CO₂ is partly absorbed by the oceans causing the increase in free hydrogen, which causes a drop in pH. Carbonate concentrations, necessary for reef-building organisms to build their calcareous shells simultaneously decrease. Plants are able to sequester CO₂ from the environment and produce oxygen and sugars during photosynthesis. Since 1850 the pH decreased from 8.2 to 8.1. If emissions are not drastically reduced, the pH will further drop according to the future scenario RCP8.5. Aggressive reductions may result in a trajectory visualized in RCP2.6.

Gattuso et al. (2015) reviewed how the ecology of the oceans, as well as their geophysics and chemistry will likely be affected in future scenarios. Two contrasting trajectories are predicted with the ‘Representative Concentration Pathways’ (RCP) scenarios of RCP8.5, where current high-emissions will proceed and RCP2.6, a stringent CO₂ emission scenario with aggressive reductions. The RCP trajectories are consistent with the Copenhagen Accord constraining the mean global temperature increase below 2°C within the 21st century. In both scenarios, key marine organisms and ecosystems will face high risk of impacts well before 2100.

Ecological impacts are occurring globally across all latitudes and require an immediate and substantial reduction of CO₂ emissions in order to prevent massive and irreversible impacts on ocean ecosystems. Global attention has already been shifted towards addressing this issue, reflected in a great number of reported scientific studies focused on CO₂ emission (Liaaskas et al., 2000; Sun, 2000; Wang et al., 2017; Wang and Li, 2019; Yoro and Daramola, 2020), remediation (Benhelal et al., 2013; Hasanbeigi et al., 2012; Jeong et al., 2018; Manan et al., 2017; Usubharatana
et al., 2006) and impacts (Alam Hossain Mondal et al., 2011; Dong et al., 2019; Mardani et al., 2019; Zheng et al., 2019). In recent years, more scientific articles estimated the consequences of OA on different marine organisms and ecosystems.

**VOLCANIC CO₂ VENTS AS NATURAL LABORATORIES**

How acidification impacts marine organisms is mainly studied using three strategies: aquarium experiments, mesocosm experiments and *in situ* experiments in natural CO₂ vent sites. In some older OA studies in aquarium conditions, acidification was induced by adding acids (such as HCl) to aquaria and exposing marine organisms to these acidified conditions (e.g. Lesniowski et al., 2015; Winans and Purcell, 2010). More recent aquarium experiments tend to expose marine organisms to elevated CO₂ concentrations, by injecting CO₂ gas into aquaria and thus increasing the partial pressure of CO₂ (pCO₂) in the seawater (e.g. Bates and Bell, 2018; Castillo et al., 2017; Enzor et al., 2017; Hernroth et al., 2011; Li et al., 2015; Takahashi and Kurihara, 2013). Carbon dioxide manipulations have also been performed in larger scale experiments such as micro-, meso or macrocosms (Jin et al., 2015; Moulin et al., 2015; Valles-Regino et al., 2015). These are experimental units, which are designed to include important components and processes occurring in a whole ecosystem (Draggan and Reisa, 1980). The prefix micro, meso or macro formulates the size of these experimental units, with arbitrary limits of < 1 m³, 1 – 1000 m³ and > 1000m³ volume, respectively (Bloesch, 1988).

OA experiments should recapitulate realistic pCO₂ values ranging between 180 and 350 ppm for ‘present-day’ (~pH 8.1) and between 450 and 1000 ppm (~pH 7.8) for acidification conditions (McElhany and Shallin Busch, 2013; Riebesell et al., 2011). However, aquarium and mesocosm studies are mostly carried out at short time scales, exposing marine organisms to acute acidification conditions. This limits their suitability in providing robust information on the acclimatization and adaptation processes of organisms to long-term progressive OA (McElhany and Shallin Busch, 2013).

Observational OA studies in hydrothermal or other natural underwater CO₂ vent sites, provide the possibility to perform natural experiments for the exploration of gradients in ocean chemistry (Figure 1.2). Certain CO₂ seeps include possible spatial or temporal confounding factors that should be minimized, which are independent of seawater carbonate chemistry. For instance, some volcanic vent sites do not only emit carbon dioxide, but also toxic gases such as methane and sulfide (H₂S), or produce heat or other parameters. Via cascading effects, these factors may affect the physiology and performance of organisms. Therefore, the selection of suitable vent and control sites, is of crucial relevance when performing observational studies in the field. These *in situ* experiments provide great advantages, allowing to explore long-term adaptations of marine organisms under chronic effects of low pH, providing a realistic picture of the impact of OA on single species and on the ecosystem (Riebesell et al., 2011).
Few vent areas are reported to have almost exclusively CO$_2$ emissions and represent suitable underwater seeps for OA studies. These vent systems include Columbretes Islands in Spain, Faial in Azores, Papua New Guinea and Ischia in Italy (Figure 1.2). In these underwater volcanic vent systems, a constant bubbling of predominantly CO$_2$ creates a decrease in pH similar to near-future OA scenarios predicted for the end of this century (Caldeira and Wickett, 2005). As natural laboratories, they are useful to forecast ecosystems’ fate in future OA scenarios (Hall-Spencer et al., 2008).

Figure 1.2: CO$_2$ vent systems used as ‘natural laboratories’ for ocean acidification studies. Adapted from González-Delgado and Hernández (2018).

The island of Ischia sits on the western margin of the Phlegraean Volcanic District and releases 15 kg of CO$_2$ s$^{-1}$ over 46 km$^2$ (Brown et al., 2008; Pecoraino et al., 2005). The island has been volcanically active for 150 thousand years (Gillot et al., 1982); volcanic vents release carbon dioxide through open conduit craters or fumaroles. Diffuse degassing from soils, such as from underwater sediments result in visible CO$_2$ bubbling (Figure 1.3). The CO$_2$ vents of Ischia are widely studied as natural laboratories to investigate the long-term effects of OA on different marine taxa, such as fish (Mirasole et al., 2021), sea urchins (Foo et al., 2018; Migliaccio et al., 2019; Nogueira et al., 2017), annelids (Del Pasqua et al., 2019; Munari et al., 2022; Ricevuto et al., 2016, 2015; Valvassori et al., 2019; Wäge et al., 2018), bryozoans (Lombardi et al., 2011; Rodolfo-Metalpa et al., 2010), isopods
(Turner et al., 2016), corals (Biagi et al., 2020; Rodolfo-Metalpa et al., 2011; Teixidó et al., 2020) and sponges (Goodwin et al., 2014; Vitale et al., 2020).

**Figure 1.3: CO₂ gas emission within *Posidonia oceanica* leaves of the Castello Aragonese vent in the island of Ischia.** Picture from Mirasole et al. (2021); Photo Credit: Pasquale Vassallo.

**WINNER AND LOSER TAXA IN FORECASTED FUTURE ACIDIFIED SCENARIOS**

OA is predicted to drive ecological changes: Marine ecosystem communities and trophic structures are predicted to lose complexity, with increasing dominance of primary producers and their lower-order consumers. While invasive non-native algal species may highly benefit under OA, higher-order consumers collapse (Hall-Spencer et al., 2008; Vizzini et al., 2017). The availability of structurally complex corals were observed to reduce, along with many macroinvertebrate groups, including crustaceans and crinoids in acidified environments (Fabricius et al., 2013). In fish, an interference in sense sensitivity under near-future CO₂ levels is predicted, manifested by neurosensory and behavioural alterations and impaired otolith growth (Heuer et al., 2014). The abundance of piscivorous fish further declines in conjunction with declining coral abundance (Bell et al., 2018b). Dramatic changes were also observed for biofouling communities, which face dramatic reductions in groups with calcareous exoskeletons, in contraposition with increasing dominance by soft-bodied biofoulers, like ascidians and sponges (Peck et al., 2015). Qualitative models which explored ecosystem-level outcomes, confirm these trends for near future scenarios, and suggest simplified benthic systems dominated by sponges and macroalgae (Bell et al., 2018b).

Different marine taxa are predicted to win or lose across a range of future OA scenarios. With decreasing carbonate availability, marine calcifiers and shell-building organisms are severely threatened. These include reef building corals, bivalves, echinoderms, bryozoans, gastropods,
crustose coralline algae and other calcareous organisms (Andersson and Gledhill, 2013; Doney et al., 2009).

The decrease in carbonate bioconstructions diminishes ecosystems structural integrity, which is necessary to support overall benthic biodiversity (Andersson and Gledhill, 2012; Kleypas and Yates, 2009). Little is known about the effect of lowered pH on the survival and physiology of non-calcifying organisms, such as soft corals or sponges, that are abundant all over the world’s oceans and constitute an important and diversified component of benthic marine ecosystems (Maldonado et al., 2012; Reverter et al., 2022). Trends in resilience vary drastically even among closely related taxa, at times exhibiting opposing responses to decreasing pH (Gambi et al., 2016). While generally an increase in the abundance of filter feeders and herbivores were observed, sessile polychaetes, both calcifying and non-calcifying species, disappeared in extreme low pH zones (Gambi et al., 2016). The decline of calcifying organisms under low pH, could favour the dominance of non-calcifying species that in many scenarios are predicted as ‘winners’ of future climate change scenarios. Among them, marine sponges have been predicted to thrive in remarkable abundances under future ocean acidification scenarios (Goodwin et al., 2014; Morrow et al., 2015).

Few OA studies consider marine sponges Figure 1.4. Between 2000 and 2013, most published articles studied calcifying cnidarians and molluscs, followed by fish, echinoderms, and crustaceans. In contrast, a notably low number of studies has been performed on marine worms and sponges, suggesting the need to conduct further studies on these ecologically important groups.

![Figure 1.4: Number of scientific articles related to OA effects for different marine phyla. Graph from Heuer and Grosell (2014).](image-url)
Marine sponges as model ocean acidification winner taxa

Sponges (Porifera), the most ancient multicellular animals on Earth, have four classes - Calcarea, Hexactinellida, Homoscleromorpha, and Demospongiae (Van Soest et al., 2012). Evolutionarily sponges likely derive from ancestral choanoflagellates ('choano' means collar), which are single-celled, colonial eukaryotes and the sister group of animals (Laundon et al., 2019; Maldonado, 2004). While the body structure of sponges is mostly stabilized by silicious spicules and collagen fibers, calcareous sponges rely on calcium carbonate structures (Hartman and Goreau, 1975).

Figure 1.5: Sponge body plan (Hentschel et al., 2012). A) A schematic overview of a typical demosponge. B) Internal demosponge structure. Sea water enters through small inhalant openings (ostia) into the sponge body due to a flow created by the beating of flagellated choanocyte cells. Filtered water is then discharged via the exhalant opening (osculum). The choanocytes capture food particles in the sea water and microorganisms are transferred to the inner mesohyl, where they are engulfed by archaeocyte cells. Symbiotic microbial communities are distributed across the mesohyl, as well as siliceous spicules, which provide structural support.

Three distinct cell layers determine the basic sponge body plan (Bergquist, 1978): the pinacoderm is the external epithelium and isolates the sponge from the external environment by a single-celled layer (pinacocytes). The choanoderm, the internal epithelium, is composed of flagellated collar-cells (choanocytes), which induce a water flow through the sponge for filter-feeding activity. The mesohyl is located between the choanoderm and pinacoderm and contains mobile cells, microbial symbionts and skeletal elements such as spicules (Figure 1.5).

This body plan supports the lifestyle as a sedentary, suspension-feeding animal (Bergquist, 1978). Upon their flagellar movement, seawater enters through the small inhaling openings (ostia) and flow through the aquiferous canal system, where choanocyte-associated collar filters retain food particles, e.g. bacteria and picoplankton. Seawater is then released through the sponge’s exhalant oscula into the surrounding environment. This simple and effective lifestyle facilitated sponges to survive even historical mass extinction events, and to thrive within diverse benthic ecosystems until
today. It can be hypothesized that these aspects might have favoured sponges towards an increased resilience under changing ocean chemistry scenarios (Bell et al., 2018a; Hentschel et al., 2012; Van Soest et al., 2012). Sponges are considered as winner taxa of future climate change scenarios, not only because of their efficient filter-feeding lifestyle, but also because of their close symbiotic association with highly diverse and complex microbial communities (Schmitt et al., 2012; Webster et al., 2010). The sponge host and its microbes form together one functional unit which is defined as ‘holobiont’ and represents one of the most diverse biological functional systems across the marine environment (Pita et al., 2018). Depending on their microbial load, high-microbial abundance (HMA) species have up to $10^8$ to $10^{10}$ microbes/g sponge tissue, comprising up to 20% – 35% of the sponge biomass, whereas low-microbial abundance (LMA) species harbour approximately $10^5$ to $10^6$ microbes/g sponge tissue (Erwin et al., 2015; Hentschel et al., 2012, 2006).

Sponge-associated microbial symbionts improve holobiont performance by providing a wide range of functional roles such as the participation in key metabolic processes, such as nitrogen, carbon and sulfide metabolism, as well as amino acid and cofactor synthesis, the production of beneficial secondary metabolites, vitamin synthesis and biochemical transformations of nutrients or waste products, finally supporting niche expansion (Hentschel et al., 2012; Siegl et al., 2011; Taylor et al., 2007; Webster and Thomas, 2016). Some cyanobacterial symbionts protect sponges by providing effective protection against UV radiation, allowing sponges to thrive in shallow waters (Glover, 1985). But also, symbionts profit from a protected niche within their sponge host and a constant nutrient supply as a result from constant filter-feeding activity. The accumulation of sponge metabolic waste products, such as ammonia, further supplies microbes with a scarce element in marine environments, nitrogen (Hentschel et al., 2012).

Another evolutionary advantage is that sponges can mostly switch between asexual and sexual reproduction to increase population sizes. During asexual reproduction (fragmentation, budding or gemmulogenesis) sponge tissue can break away from the mother sponge, attach to a new substratum and form a new sponge. Under unfavourable environmental conditions, some sponges can become dormant even for long periods. Such dormancy is often associated with increased asexual reproduction (Ereskovsky, 2018). Several sexual reproduction strategies are known to occur in sponges. During ‘viviparous’ reproduction, sperm cells are released by a sponge through the oscula into the surrounding seawater. Sperm cells then swim and eventually enter through the ostia of a receptor sponge into the internal mesohyl, where they can meet mature oocytes and fertilize them. The fertilized eggs develop into larvae that are exhaled to the environment. Free-swimming planula larvae swim until they find a suitable substrate, were they attach, settle and become an adult sponge (Figure 1.6). Mostly hermaphroditic brooder sponges perform this viviparous reproduction strategy, where larvae generally develop internally, and are then released into the water column (Ereskovsky, 2018; Riesgo et al., 2014).
In oviparous (gonochoric) sponges, unfertilized eggs or zygotes and sperm cells are released into the water. Fertilization and embryonic development take place externally, to produce swimming larvae that colonize appropriate substrata and develop into adult sponges. However, in some oviparous demosponges, for example, *Cliona celata* and *Chondrosia reniformis*, an internal fertilization occurs (Ereskovsky, 2018). Similar to cnidarians, hermaphroditism and viviparity as well as gonochorism and oviparity are strongly correlated in sponges (Kerr et al., 2011).

During reproduction, microbial symbionts can be transferred from the mother sponge to their offspring via eggs (in oviparous sponges) or within larvae (in viviparous sponges) (Bright and Bulgheresi, 2010; Ereskovsky et al., 2005; Mira and Moran, 2002; Schmitt et al., 2008). This so-called ‘vertical symbiont transmission’ (Schmitt et al., 2008) is crucial to maintain key microbial functions across generations. Sponges further shape their microbial communities by acquiring microbes from the surrounding environment – horizontal symbiont transmission (Heath and Tiffin, 2009; Nyholm and McFall-Ngai, 2004). This strategy allows to dynamically reshape associated microbiomes under changing environmental conditions.

Up to date, few studies have addressed the impact of OA on sponges and their associated microbial communities (Bell et al., 2018a; Morrow et al., 2015; Ribes et al., 2016). In natural volcanic seeps in
Papua New Guinea, researchers found decreased coral populations, coinciding with losses in associated symbionts (*Endozoicomonas*). At the same time, highly abundant sponge species *Coelocarteria singaporensis* and *Cinachyra spp.* showed significantly higher *Synechococcus* abundances at the vent site. These symbionts may provide a nutritional benefit for their hosts, facilitating growth and survival in lower pH conditions. Their data showed that responses to OA are species-specific, and that the stability and flexibility of microbial partnerships may have an important role in contributing to the success of some hosts (Morrow et al., 2015). Another study with Mediterranean sponges exposed to OA conditions for 66 days, showed no variation in overall microbial abundance, richness or diversity, but reported the ability of sponges to acquire new microbes from the environment through horizontal transmission (Ribes et al., 2016). Sponge growth rates were positively correlated with the ability of sponges to modulate their microbiome communities, providing further support that flexible microbial communities are important for sponge tolerance to OA (Ribes et al., 2016). The role of microbiomes in supporting resilience of sponge holobionts under OA requires, in any case, further investigation (Bell et al., 2018a).

Sponges and their associated microbiome are key players in ecosystem stability and need increased attention to predict the health and integrity of our future oceans (Reviewed by Pita et al. (2018). According to the principles of a ‘nested ecosystem’, already small perturbations within the sponge microbiome can result in physiological changes for the entire sponge holobiont. Via cascading effects consequences can reach out to community and ecosystem level (Figure 1.7). Microbial metabolism provides nutrients to their sponge host via several strategies, including chemoautotrophy and photoautotrophy. Sponges in turn can represent a food source for some predators and compete for space with other reef building organisms. Microbial-mediated functions can scale up to influence marine ecosystems by contributing to overall primary productivity, biogeochemical nutrient cycling and benthic food webs (Pita et al., 2018).

One of the key mechanisms, fuelled by the high filtering capacity of sponges, is the so-called ‘Sponge Loop’. Sponges filter dissolved organic matter (DOM) from the seawater, where microbial symbionts play a key role in DOM assimilation and subsequent DOM degradation (De Goeij et al., 2013; Hudspith et al., 2021). DOM is then transformed into particulate organic matter, which is released back into the environment, fuelling the surrounding ecosystem with a (to higher organisms) profitable nutrient form.
Figure 1.7: Nested ecosystem concept. Microbes associated with the holobiont perform key functions (coloured arrows) such as photosynthesis, defense, predation, the sponge loop, nitrification and competition. Via cascading effects microbial functions are able to influence community and ecosystem structures. Schematic representation from Pita et al. (2018).

**Thesis Outline**

This thesis addresses one of the current hot topics in climate change research, especially focusing on ocean acidification (OA) and possible outcomes for our future oceans. Sponges are often seen as winner of future climate changes scenarios (Bell et al., 2018a), but responses are species-specific and several gaps of knowledge persist. Sponges may develop increased tolerance due to dynamical changes in their microbial communities (Bell et al., 2018a; Ribes et al., 2016), but it is unclear whether the HMA or LMA status impacts the success of a sponge species for acclimatization and adaptation. In this thesis, natural volcanic CO₂ vents were used as natural laboratories to investigate the effect of OA on long-term adapted sponge communities, targeting two Mediterranean species: the HMA – high-microbial abundance sponge *Chondrosia reniformis* and the LMA – low-microbial abundance species *Spirastrella cunctatrix*. Species-specific responses and different adaptive traits to OA are highlighted using two different omics-approaches, 1) Microbial community characterization via NGS and 2) NMR-based metabolomics.

Short-term aquarium experiments suggested that the HMA sponge *C. reniformis* lacks the ability for microbial restructuring, suggesting a poor tolerance under OA (Ribes et al. (2016). For the LMA sponge *S. cunctatrix* no targeted acidification studies were performed yet. Chapter II focuses on symbiotic microbial community responses of sponge holobionts, comparing individuals from the
natural CO\(_2\) vent site with lowered pH off Ischia and a comparative control site with “normal” pH. Dynamics of sponge-associated microbiomes revealed different trends in microbial responses of HMA and LMA holobionts to OA. Along with traditional biodiversity tools, core microbiomes and differentially abundance analyses are presented. Moreover, I discuss potential microbial functions, which may potentially participate in holobiont resilience to cope with OA as environmental stressor and increase overall organismal performance. Three Indicators for a stress-induced dysbiosis are discussed for the LMA species, which might have impacted morphological integrity.

To date, no metabolomics studies of \textit{C. reniformis} or \textit{S. cunctatrix} were performed. Chapter III presents the first NMR spectra and metabolic shifts for both sponge species in response to OA. First, the general chemical composition is described based on identified metabolites from one- and two-dimensional NMR spectra using polar crude sponge extracts. Trends of metabolite variations in CO\(_2\) vent conditions are predicted using multivariate OPLS-DA regression analyses (i.e. Orthogonal Projections to Latent Structures Discriminant Analysis). Changes in relative metabolite concentrations were statistically validated and possible consequences are discussed in the context of the sponge host and its associated microbiome.

Sponges are critical players in nutrient cycling of benthic ecosystems, but little is known about the specific biomechanisms that take place. Seawater properties are altered as a result of filter-feeding activities and the comparison of inhaled (In) versus exhaled (Ex) seawater fluxes can reveal which components are being produced or consumed. Current sampling methods, such as the VacuSIP (Morganti et al., 2016), have several limitations such as intensive manual work for scuba divers, contamination by ambient water and insufficient sample volumes for comprehensive down-stream analyses. Chapter IV describes a novel methodology to sample inhaled and exhaled (InEx) seawater fluxes from filter-feeders using an underwater peristaltic pump system – the ‘PeriSIP’. This technique was developed using \textit{C. reniformis} as study animal, and provides pilot results in biochemistry parameters from aquaria and \textit{in situ} experiments. Final aim is to provide an easy-to-use sampling device that introduces a robust technique, to advance our understanding of filter-feeding activities and their role for marine ecosystems.

Finally, the concluding remarks summarize the key results obtained from this PhD project and aims at integrating microbiome and metabolomics results to depict a broader picture of sponge responses to OA. Connected with the novel PeriSIP methodology, the study of biogeochemical fluxes, such as filter-feeders’ InEx fluxes, could be applied for future OA studies. Further \textit{in situ} research of whole filter feeding assemblages is proposed to better understand local chemical changes and consequences for ecosystems, the fate of nutrient recycling, and the role of sponges and other filter-feeders in such processes.
Main hypothesis and aims examined in this thesis are:

1) OA triggers the restructuring of the microbiomes of the HMA sponge *C. reniformis* and the LMA sponge *S. cunctatrix*, through processes such as symbiont acquisition and depletion, and symbiont shuffling. This restructuring is expected to confer adaptive traits to the sponges under OA stress.

2) OA induces microbial dysbiosis in *S. cunctatrix*, resulting in impaired physiological functions that may impede its ecological success.

3) Functional redundancy of sponge-associated symbionts is beneficial to maintain organismal health under microbiome restructuring.

4) The exposure to an acidified environment alters the metabolomic profiles of *C. reniformis* and *S. cunctatrix*, as compared to their respective control conditions. Aim is to identify and quantify metabolites that may be responsive to OA, providing insight into the potential impacts of changing ocean conditions on these sponge species.

5) The metabolomic profile of the LMA sponge exhibits sensitivity to OA, while the HMA sponge's metabolome displays a higher degree of constancy. Notably, the resilience of the HMA sponge's metabolome is linked to the functional redundancy of its microbial community.

6) The ‘PeriSIP’ is a novel technology and methodology that enhances the accuracy and efficiency of *in situ* sampling of inhaled and exhaled seawater of filter-feeding organisms. This technology is anticipated to offer improved sampling capabilities for studying filter-feeding organisms in their natural habitats.


CHAPTER II

HOLOBIONTS IN A CHANGING OCEAN –
DO MICROBES PLAY A ROLE IN ORGANISMAL ADAPTATION TO OCEAN ACIDIFICATION?
ABSTRACT

The impact of Ocean Acidification (OA) on marine biochemistry is profound, resulting in a net loss of biodiversity. Benthic organisms must develop adaptive strategies to cope with OA. Marine sponges are often forecasted as winner taxa, yet, we lack mechanistic understanding. The holobiont concept aims at assessing the sponge host and its symbiotic microbiome as a metaorganismal unit, when evaluating health, adaptive traits and resilience to environmental challenges. Sponges can be categorized into high and low microbial abundance (HMA, LMA) species, and may adopt divergent strategies to maintain homeostasis and fitness under changing conditions.

In this study, microbial shifts are compared across the HMA sponge *Chondrosia reniformis* and the LMA sponge *Spirastrella cunctatrix* from a natural CO$_2$ vent with lowered pH values, and a control site with “normal” pH off Ischia Island. Microbial diversity and composition changed for both species in response to OA. Shifts in core microbial communities were detected, showing an increased number of core taxa for *S. cunctatrix* and a generally more diverse core microbiome for *C. reniformis*. *S. cunctatrix* showed, along with morphological impairment, signs for a putative stress-induced dysbiosis, manifested among the vent associated microbiome by 1) increases in alpha diversity, 2) higher beta diversity dispersion and 3) a shift from sponge related microbes towards seawater microbes. The HMA sponge showed no significant morphological changes at the vent site. It is hypothesized that *C. reniformis* may attain higher functional redundancy, thanks to a more diverse microbiome, which could promote acclimatization to environmental changes. Differential abundance analyses revealed consortia of vent and control site associated bacterial features, with broad metabolic functions, including the participation in carbon, nitrogen and sulfur-metabolism supporting host nutrition and biogeochemical cycling.

Host and microbially mediated responses to OA are supported by mechanisms that can be involved in acclimatization strategies, which should be addressed in future studies. Many bacterial features have been identified potentially supporting functional convergence, likely increasing the resilience of sponges under OA.

Keywords: Ocean Acidification, Porifera, Climate Change, Microbiome, CO$_2$ vent, Sponges, Symbiosis
INTRODUCTION

SPONGES AS WINNER TAXA?

Sponges are often predicted to be winner taxa of future ocean acidification (OA) scenarios (Bell et al., 2018a), in which the actual pH of 8.1 is predicted to decrease to 7.8 by the year 2100, due to steadily increasing atmospheric CO₂ concentrations. In comparison to other benthic organisms, such as calcifying corals, sponges seem to be more tolerant to OA and are expected to obtain benefits under near-future scenarios due to reduced spatial competition, and increased productivity (Aronson et al., 2002; Bell et al., 2018b, 2018a, 2015, 2013; Colvard and Edmunds, 2011, 2011; Kelmo et al., 2014). The calcification process depends on the availability of carbonate to form calcium carbonate structures, and this process is reduced under acidified conditions (Doney et al., 2009), making calcareous organisms especially sensitive to OA. In this sense, since Porifera include relatively few species which rely on calcium carbonate components and frequently implement siliceous or collagen structures, interference on sponge skeleton formation is predicted to be minimal (Van Soest et al., 2012; Vicente et al., 2016).

Another advantageous trait of sponges is based on their flexible nutrition supporting their survival in oligotrophic environments (De Goeij et al., 2013; Hadas et al., 2009). By filter-feeding, sponges take up different forms of particulate organic matter (POM) (Hadas et al., 2009), such as detritus, free-living bacteria (Wilkinson, 1978a) and planktonic eukaryotes, including the typical prey taxa *Synechococcus spp.* (Hadas et al., 2009; Morganti et al., 2016). They are further able to take up dissolved organic matter (DOM), deriving from algal and coral waste products (De Goeij et al., 2013; Rix et al., 2017; Wilkinson and Garrone, 1980). Additionally to heterotrophic feeding strategies, sponges are able to profit from energy and nutrient supply from chemo- and photoautotrophic symbionts, which live in close associations with their sponge host (Cheshire and Wilkinson, 1991; Feng and Li, 2019; Nakagawa and Takai, 2008; Ribes et al., 2012). Rich microbial assemblages provide diverse metabolites which supply sponges with ecological advantages to withstand spatial competitors, predators and pathogens (Pawlik, 2011; Porter and Targett, 1988; Thakur and Singh, 2016). However, not every sponge species is resilient to OA at the same level (Bates and Bell, 2018; Botté et al., 2019; Fabricius et al., 2011), and still it is unclear to which extent sponge-associated microbiomes are involved in possible mitigation processes.
THE HOLOBIONT CONCEPT

Sponges form intimate associations with diverse microorganisms (Moitinho-Silva et al., 2017a). These partnerships are essential to sponge health and survival (Pita et al., 2018; Webster and Thomas, 2016) and cover diverse interactions with bacteria, Archaea, Fungi, protists, and viruses (Webster and Taylor, 2012). According to the holobiont concept, introduced 1990 by the evolutionary theorist Lynn Margulis (Margulis, 1990), sponges and their associated microbiome (Moitinho-Silva et al., 2017a) are considered as metaorganismal systems forming one functional biological unit (Bordenstein and Theis, 2015; Rohwer et al., 2002). Evaluating the stability and dynamics of these systems, can reveal the contributions of the microbiome to the maintenance of a healthy holobiont and its response to environmental changes, supporting resistance and resilience to disturbances (Costello et al., 2012; Fierer et al., 2012; Pita et al., 2018; Sommer et al., 2017).

Symbiosis became an evolutionary ‘prodigy’ for survival across diverse environments seeking for new habitats (Bronstein et al., 2004). As Darwin emphasized, the struggle for existence includes the dependence of one organism on another (Darwin, 1859). This is of special importance for sponges in colonizing oligotrophic marine environments, where microbial symbionts promote niche expansion though nutrient contribution to host metabolism (Stanley, 2006). Coral reef sponges often host cyanobacteria as photosynthetic symbionts (Erwin and Thacker, 2007; Rützler, 1990) and benefit from supplemental nutrition (Arillo et al., 1993), nitrogen fixation (Wilkinson and Fay, 1979), production of secondary metabolites (Flatt et al., 2005) and UV protection (Regoli et al., 2000). Sponges inhabiting extreme environments, such as deep-sea hydrothermal vents, benefit from nutritional contribution by chemosynthetic bacteria (Dubilier et al., 2008; Georgieva et al., 2020). As primary producers, chemolithotrophs oxidize sulphur or methane to obtain energy from the conversion of inorganic carbon molecules (CO$_2$ or methane) into organic matter, which in turn is supplied to their sponge host. The required energy to allow this synthetic pathways derives from the oxidation of inorganic compounds, such as sulphide or methane (Dubilier et al., 2008). Several strategies of microbial symbioses thus represent major sources of primary productivity and nutrient cycling in sponge holobionts (Diaz and Rützler, 2001; Mohamed et al., 2008).

In addition to supplying their hosts with nutrients (Arillo et al., 1993; Wilkinson and Fay, 1979) microbial symbionts can enhance growth and competitive abilities of sponges within benthic communities (Erwin and Thacker, 2008; Flatt et al., 2005; Regoli et al., 2000; Thacker, 2005; Wilkinson, 1987, 1983). Hosting symbionts allows sponges to access products of microbial metabolism that are beneficial for sponge physiological performance and reproductive success (Freeman et al., 2014). For example, resident bacterial community members provide their sponge host with essential vitamins, such as vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B7 (biotin), and vitamin B12 (cobalamin) (Fan et al., 2012; Hallam et al., 2006; Liu et al., 2012; Siegl et
al., 2011; Thomas et al., 2010), which cannot be produced by eukaryotic hosts and need to be acquired from other sources (Roth et al., 1996). In other symbiotic relationships, microbial symbionts produce light absorbing pigments such as melanin, which due to their antioxidant properties protect sponge cells from photo-toxicity, allowing sponges to withstand increased UV radiation (Vijayan et al., 2017). Sponges are able to accumulate bacterial natural products, which allow different chemical defense strategies, such as feeding deterrent compounds which protect sponge hosts from predatory fish (Becerro et al., 2003; Pawlik et al., 1995; Schupp et al., 1999), compounds with anti-fouling activity (Becerro et al., 1994; Martín and Uriz, 1993; Stowe et al., 2011; Thompson et al., 1985) and secondary metabolites which suppress the growth of competing invertebrates (Porter and Targett, 1988; Sullivan et al., 1983; Turon et al., 1996).

New metabolic capabilities that emerge as a result of evolutionary forces, support the stability of host-symbiont associations (Margulis, 1993). Sponges predominantly harbour sponge-specific microorganisms and present a selective niche for associated symbionts (Simister et al., 2012a). The interplay of the host and its symbiotic consortia form an evolving biological community that changes throughout time (Moya et al., 2008). Especially under challenging environmental shifts such as OA, sponges may develop increased tolerance due to a certain degree of dynamical changes in their microbial communities (Bell et al., 2018a; Ribes et al., 2016). On the other hand, maintaining a high degree of stability of their associated microbiome also results in constitutive benefits (Pita et al., 2013a) across spatial and temporal scales (Erwin et al., 2012; Pita et al., 2013b; White et al., 2012).

**HORIZONTAL AND VERTICAL SYMBIONT TRANSMISSION**

Sponges shape their microbial communities by acquiring symbionts through two different strategies – horizontal and vertical symbiont transmission (Figure 2.1). Vertical transmission implies that symbionts are inherited through parental gametes or brooded embryos (Buchner, 1965; Mira and Moran, 2002), while horizontal transmission usually occurs through acquisition from the surrounding environment (Heath and Tiffin, 2009; Nyholm and McFall-Ngai, 2004). There are generally two vertical transmission strategies from parental sponges into their offspring. Symbionts may be either transferred within eggs in oviparous sponges, or within larvae in viviparous sponges (Bright and Bulgheresi, 2010; Ereskovsky et al., 2005; Schmitt et al., 2008). Symbionts in some oviparous sponges, are collected from the mesohyl by amoeboid nurse cells, to be then transported and transferred to oocytes for vertical transmission. Other oviparous species lack vertical symbiont transmission into gametes or embryos, while relying on horizontal symbiont transmission from the environment to the juveniles of each new generation (Maldonado, 2007). Only for few viviparous sponges evidence of vertical transmission was provided (Kaye and Reiswig, 1991; Ereskovsky and Boury-Esnault 2002). Some viviparous species are able to transfer symbionts directly into gametes,
while other species rather accumulate symbionts around developing embryos. Microbial cells enter the embryotic cells during cell division by accumulating around the cleavage furrow of early embryotic cells. Microbes then further proliferate in the central cavity of the free-swimming larva (Maldonado, 2007).

A mixture of both strategies is termed ‘leaky vertical transmission’, whereby symbionts are both acquired from the surrounding environment as well as passed vertically via gametes or embryos (Thacker and Freeman, 2012; Vrijenhoek, 2010). This transmission mode may represent the most common evolutionary strategy for the acquisition of microbial communities in sponges (Thacker and Freeman, 2012). Indeed, both transmission modes present advantages as well as challenges while shaping holobionts under changing environmental parameters (Ewald, 1987). One advantage of the parental derived symbiont transmission is the assurance that the sponge offspring immediately obtains the necessary symbionts for optimal growth (Vrijenhoek, 2010). But after several generations this potentially reduces genome sizes of obligate symbionts, leading to a loss of metabolic capabilities (Moran, 2002). Furthermore, offspring that disperses into spatially distant
habitats or need to adapt to changing environmental parameters may be associated with not optimal symbiont strains and would rather profit of a natural selection for locally optimal and genetically diverse strains of symbionts adapted to the environment (Vrijenhoek, 2010; Won et al., 2003). However, horizontal transmission also implies risks for holobiont stability. Besides evolving ‘cheater’ symbionts that exploit the host (Frank, 1996), also pathogens can potentially employ the same pathways and infect a host. Conclusively, a leaky vertical transmission mode might be most beneficial by combining the benefits of purely vertical and horizontal modes of symbiont acquisition (Vrijenhoek, 2010).

**HMA and LMA Sponges**

Sponges can be classified into two major categories according to the abundance of associated symbiotic microorganisms (Gloeckner et al., 2014). High-microbial abundance (HMA) species harbour very dense and diverse microbial consortia (Reiswig, 1974; Vacelet and Donadey, 1977; Wilkinson, 1978b) containing between $10^8$ to $10^{10}$ microorganisms per gram sponge tissue, comprising 20% - 35% of their total biomass (Hentschel et al., 2012; Reiswig, 1981; Webster et al., 2001). Low-microbial-abundance (LMA) sponges instead, contain between $10^5$ to $10^6$ bacteria per gram tissue in lower taxonomic diversity than HMA sponges (Hentschel et al., 2006). LMA sponges are usually mainly dominated by few bacterial taxa belonging to the phyla Proteobacteria (Alpha-, Beta-, and Gamma-) or Cyanobacteria (genus *Synechococcus*), whereas HMA microbiomes often span a wide-range of dominant taxa belonging to Proteobacteria, Chloroflexi, Acidobacteria, Actinobacteria, candidate phylum Poribacteria and other phyla (Gloeckner et al., 2014).

Different processes during vertical transmission shape these patterns of typical HMA and LMA microbiomes. While HMA sponges contain dense bacterial assemblages at the larval centre, most LMA sponge larvae are largely free of microbes, but there are exceptions (Ereskovsky and Tokina, 2004; Gloeckner et al., 2013a, 2013b; Maldonado, 2007; Schmitt et al., 2007). Claude Lévi (1976) firstly showed vertical transmission taking place in the oviparous HMA sponge *Chondrosia reniformis*. The microbial component from vertical acquisition in HMA sponges (Bright and Bulgheresi, 2010; Gloeckner et al., 2014), is complemented by horizontally transmitted symbionts from the seawater (Schmitt et al., 2008; Webster et al., 2010). Analogously, yet in lesser proportions than HMA species, also oviparous LMA species, like *Spirastrella cunctatrix* have demonstrated to acquire symbionts from their parental counterparts (Riesgo et al., 2014). Although some sponge species rely more on environmental acquisition of symbionts than vertical transmission, there is no clear pattern based on the HMA-LMA status.

HMA-LMA dichotomy is further correlated with morphological differences. Compared to LMA species, HMA sponges have a denser mesohyl and complex aquiferous systems that allow a 52-94% slower water filtration rates across long and narrow water channels (Vacelet and Donadey, 1977;
Weisz et al., 2008). Since HMA sponges have much smaller choanocyte chambers than LMA species, they would need to invest more energy to increase filtering pumping rates (Boury-Esnault and Vacelet, 1990). Their morphological structure increases their surface area and contact time between seawater and sponge tissue, to efficiently feed on POM, exchange DOM and inorganic material (Vogel, 1994). As a consequence, DOM and metabolic end-products accumulate by sponge and microbial metabolism, creating sponge internal microenvironments that foster microbial diversification (Weisz et al., 2008).

To supply their nutritional needs, LMA sponges move larger quantities of seawater through their porous tissues (Boury-Esnault et al., 1990; Rützler et al., 1990), allowing them to rapidly and efficiently filter POM. Their metabolic cost is minimized through wider aquiferous systems with shorter channels and large choanocyte chambers (Riisgård et al., 1993), representing a different successful nutritional strategy (Weisz et al., 2008). The two different body plans of HMA and LMA sponges likely resulted from evolutionary selection based on two different trade-offs, one morphology suited to accumulate symbiotic microorganisms, and the other to pump and filter seawater at high rates (Weisz et al., 2008).

Based on their high microbial abundance, HMA sponges profit from biochemical capabilities of their associated bacteria, Archaea and Fungi (Hentschel et al., 2006; Weisz et al., 2007), and are often proposed to be better adapted to utilize DOM (Hoer et al., 2018; Morganti et al., 2017; Reiswig, 1981; Ribes et al., 1999; Wooster et al., 2019). HMA sponges can also directly uptake DOM, without the need of microbiome mediation (Hudspith et al., 2021). A recent study, analysing DOM transformation in three different Caribbean HMA and LMA species showed that only HMA, but not LMA species, took up dissolved metabolites and changed the composition of the exhaled seawater.Authors suggested that microbial symbionts are crucial in DOM uptake and that the resulting metabolites likely serve as food source or as building blocks for secondary metabolites (e.g., chemical defences), providing selective advantages for the overall holobiont (Olinger et al., 2021). However, other studies have proved that some LMA sponges are also able to take up DOM at equal or even higher rates than those of HMA species (Bart et al., 2020; De Goeij et al., 2017; De Goeij et al., 2013; Mueller et al., 2014; Rix et al., 2020, 2017). In conclusion, both HMA and LMA sponges can efficiently use DOM.

Facing environmental changes, conceptual holobiont models related to microbiome and function predict higher functional redundancy and higher ecosystem stability at higher microbial diversity. Along with a greater probability of the presence of stress-resistant taxa (Konopka, 2009), compensatory dynamics between functionally similar species is supposed to be a key aspect in theoretical ecology dealing with environmental perturbations (Loreau, 2000; Tilman, 1999). Furthermore, it has been proposed that the network of interactions between microbes can provide a buffer effect against environmental change because functionally different microbes occupy a
network of complementary niches (Konopka et al., 2015). In this framework, it is very likely that HMA sponges that harbour richer microbiota, could be less affected by OA, because of the higher probability of functional redundancy and network complexity (Pita et al., 2018).

**THE CONCEPT OF THE ‘CORE MICROBIOME’**

The term ‘core microbiome’ has received increasing attention among microbial ecologists over the last decade (Neu et al., 2021). Determined by stable, consistent microbial taxa found across a defined environment, within the sponge host, these commonly occurring symbionts are likely to be critical to ecological and physiological competence (Shade and Handelsman, 2012). The long-term stability of the core microbiome is likely critical, because the persistent occurrence of beneficial microbes ensure host health and well-being (Cho and Blaser, 2012; Hartman et al., 2009; Lozupone et al., 2012; Missaghi et al., 2014; Relman, 2012). Especially for filter-feeders, high bacterial densities of a core microbiome seem to determine sponge microbiome stability, and confer host resistance against the establishment of putatively opportunistic taxa that sponges are constantly encountering through their filtering activities (Björk et al., 2018). Furthermore, it seems that the core microbiome plays key roles in resistance to changing environmental parameters. A transgenerational selective breeding experiment on tropical fish tested their cold tolerance and the role their associated microbiome plays in response to cold stress. Authors found that thermal tolerance shaped the microbiome composition and its response to stress. Especially core communities were identified to be among the most resilient symbionts in adapted cold-exposed fish (Kokou et al., 2018). Analysing the dynamics of the core microbiome within sponges thriving in vent sites can extend our knowledge about key communities, which support host resilience by forming a ‘healthy’ microbial community under environmental challenges.

Within the sponge host, members of the core microbiome with large genome sizes are potentially adapting to variable environments through increased levels of horizontal gene transfer (HGT) (Horn et al., 2016; Pita et al., 2018). Through a high number of mobile genetic elements, as well as transposases and integrons, which are necessary for genetic transfer and inclusion (Dobrindt et al., 2004; Sobecky and Hazen, 2009), core symbionts may adapt to changing environmental conditions by increasing their genetic diversity (Fan et al., 2012; Gao et al., 2014; Thomas et al., 2010; Wiedenbeck and Cohan, 2011). During the feeding behaviour of sponges, environmental food bacteria are digested through phagocytosis and environmental bacterial DNA can be set free. This possibly increases the possibility that mobile genetic elements, such as plasmids, are incorporated into the genetic repertoire of residing symbionts (Sobecky and Hazen, 2009), allowing the acquisition of novel functions without shifts in taxonomical composition (Putnam et al., 2017; Rosenberg et al., 2007).
**Volcanic CO$_2$ Vents as Natural Laboratories**

Underwater cold CO$_2$ seeps provide a natural habitat with increased CO$_2$ concentrations and can prevail for years to millennials. Such systems that maintain seawater temperature at regular ranges and lack sulphur and other potentially toxic compounds, can be used as natural laboratories to investigate long-term effects of OA at ecosystem level, and predict how holobionts may respond to future scenarios (Fabricius et al., 2015, 2014; Goodwin et al., 2014; Uthicke et al., 2013). Few natural volcanic CO$_2$ vent systems exist around the globe, and these are characterized by a constant bubbling of predominantly CO$_2$ emissions, creating a decrease in the pH similar to that predicted for the end of this century (Caldeira and Wickett, 2003, 2005). Some of these systems are found around the island of Ischia (Italy), Columbretes Islands (Spain), Faial in Azores (Portugal) and in Papua New Guinea (González-Delgado and Hernández, 2018).

Ischia Island offers a particularly suitable environment to perform OA studies by providing several CO$_2$ vents (Foo et al., 2019; Hall-Spencer et al., 2008; Kroeker et al., 2013, 2011) with different pH conditions, generating a gradient of influence. Low pH zones with a decrease in surface pH from −0.14 to −0.4 pH units (relative to 1870) are used for future predictions for the year 2100 (Bopp et al., 2013; Gattuso et al., 2015) and have mean pH values depending on vent site between 7.2 and 7.8 (Teixidó et al., 2018). Other vent systems offer even more extreme acidified zones, with mean pH values of 6.68 (Teixidó et al., 2018), and can be used to estimate ecological outcomes for more utmost future scenarios predicted by 2500 (Caldeira and Wickett, 2003).

Some sponge species are more tolerant than others and show OA tolerance and a wide distribution along CO$_2$ vent systems. At the vent sites off Ischia Island rich sponge communities have been reported (Pulitzer-Finali and Pronzato, 1970; Pulitzer-Finali et al., 1976; Sarà, 1960). The species *Crambe crambe* inhabits sites even with mean pH 6.6 (Goodwin et al., 2014; Teixidó et al., 2018), while most sponge species are found at mean pH 7.8 – 7.9, including *Chondrosia reniformis*, *Spirastrella cunctatrix*, *Phorbas tenacior*, *Scalarispongia scalaris*, *Icrinia variabilis*, *Sarcotragus* sp., *Haliclona mediterranea* (Goodwin et al., 2014; Mazzella, 2021). At the vent site Upa-Upasina in Papua New Guinea a seep-tolerant species *Coelocarteria singaporensis* was found to be 40-times more abundant at the vent compared to control sites. Oppositely, the seep-sensitive species *Stylissa flabelliformis* was found at 6-times higher abundance at control sites (Fabricius et al., 2011).
**MICROBALLY-MEDIATED TRANSGENERATIONAL ACCLIMATISATION TO ENVIRONMENTAL CHANGES**

Under changing environmental conditions such as OA, microbes may contribute to transgenerational acclimatisation of reef species (Webster and Reusch, 2017). Described mechanisms involve microbial frequency shifts (symbiont shuffling), the introduction of new microbial taxa (symbiont switching) and/or horizontal gene transfer (HGT) between microbial species. While a loss of beneficial symbionts may result in a decline of host health, an increasing abundance of beneficial symbionts can support host fitness and/or environmental stress tolerance of a holobiont. Horizontal symbiont transmission can increase the functional stability of sponge holobionts to withstand future OA by modifying their associated microbiomes (Morrow et al., 2015; Ribes et al., 2016). For instance, OA mediated microbial amplification was recently reported for sponges inhabiting a CO$_2$ vent sites in Papua New Guinea. In comparison to individuals inhabiting control sites, sponges were 40-times more abundant at the vent site and harboured different microbial communities compared to individuals at corresponding control sites, e.g. increased abundances of photosynthetic microbes (Morrow et al., 2015). The ability of sponge microbiomes to be restructured in response to OA seems to be species-specific (Bates and Bell, 2018; Bell et al., 2018a; Chaib De Mares et al., 2017; Reveillaud et al., 2014; Schmitt et al., 2012; Webster and Thomas, 2016). While pH tolerant sponge species revealed variable microbiomes with compositional shifts (Kandler et al., 2018), the pH sensitive species *Stylissa massa* maintained a stable microbiome and showed significantly lower abundance at CO$_2$ vent sites. These results underline the significance of symbiont shuffling as key acclimatisation, supporting the success of sponges to withstand future OA (Kandler et al., 2018).

Symbiont switching in response to environmental changes was particularly observed in reef sponges (Webster et al., 2011), foraminifera (Webster et al., 2016) and scleractinian corals (Boulotte et al., 2016). Exposing the sponge *Rhopaloeides odorabile* to high-nutrient and low-salinity conditions over 12 months resulted in replacing native symbionts with those microbial taxa typically found in sponges inhabiting nutrient-rich environments (Webster et al., 2011). In the foraminifera *Heterostegina depressa* different temperature and pCO$_2$ levels were followed by symbiont switching of different Acidomicrobiales taxa (Webster et al., 2016). Coral holobionts were shown to be able to select upon optimal algal symbionts from their environment, i.e. thermally resistant *Symbiodinium* clades, potentially driven by preceding bleaching events (Boulotte et al., 2016). Additionally to symbiont shuffling or switching, symbiotic microbes can adapt to changing environmental parameters by genetic mutations. Compared to eukaryotes, microbial cells divide at
much higher rates and are more prone to adaptive evolution. Genetic mutation is greatly enhanced by horizontal gene transfer (Webster and Reusch, 2017).

This PhD thesis chapter aims at researching the symbiotic community composition of long-term adapted sponge assemblages inhabiting CO₂ vents and control sites off Ischia Island. The investigation of compositional shifts and presence/absence of microbial ASVs in two Mediterranean demosponge species, the HMA species *Chondrosia reniformis* and the LMA sponge *Spirastrella cunctatrix*, allow an estimation of these species experiencing symbiont shuffling and/or symbiont switching in response to OA. Microbial communities are characterized based on results from Illumina Next-Generation Sequencing, targeting the v3-v4 amplicon region of the prokaryotic marker gene for 16S rRNA. Microbial diversities, community compositions and differential abundances are obtained from different cutting edge bioinformatic tools. Final aim is to estimate the role of microbial acclimatization strategies of sponge holobionts to withstand future climate change stressors.

**STATEMENT OF CONTRIBUTION**

Long-term *in situ* logger data for light, temperature and pH were provided by Dr. Nuria Teixidó. 16S rRNA Illumina Sequencing was performed by Dr. Gwenn Tanguy at the Station Biologique de Roscoff (France) supported by EuroMarine and EMBRC. Dr. Francesca Margiotta measured concentrations of seawater nutrient at the SZN. Flowcytometry analyses were performed by Dr. Raffaella Casotti at the Flow Cytometry Facility of Stazione Zoologica Anton Dohrn (Italy).
MATERIAL & METHODS

SPONGE SPECIES

As Mediterranean representatives for HMA and LMA sponges, two common demosponges were selected - the massive HMA sponge *Chondrosia reniformis* (Figure 2.2) and the encrusting LMA sponge *Spirastrella cunctatrix* (Figure 2.3).

Figure 2.2: The HMA sponge *Chondrosia reniformis*. A) Overview of sponge colony. B) Close-up picture of sponge oscula. (Photography by Alberto Colletti).

Figure 2.3: The LMA sponge *Spirastrella cunctatrix*. A) Overview of sponge colony. B) Close-up picture of sponge oscula. (Photography by Alberto Colletti).

*C. reniformis* is a brown-grey coloured sponge with a collagenous mesohyl structure with mechanical adaptability of the bulky sponge body. It lacks endogenous spicules, but incorporates exogenous siliceous material to strengthen its outer body layer. The formation of long outgrowths from the parent body (Figure 2.2A) are likely developed due to adaptive strategies related to environmental factors, asexual reproduction or locomotor phenomena (Bonasoro et al., 2001). S.
**cunctatrix** is a dull salmon-brown encrusting sponge species with large visible exhalant canals (Figure 2.3B) and a thin body layer. Embedded in collagen fibrils, the skeleton consists of siliceous spicules (tylostyles) and ectosomal layers of spirasters. Both species are able to vertically transmit symbionts across generations and reproduce via oviparous reproduction cycles (Lévi, 1976; Riesgo et al., 2014). For *C. reniformis* a high proportion of vertically transmitted symbionts is described, whereas *S. cunctatrix*, seems to rely more on environmentally acquired symbionts (Ribes et al., 2015).

**STUDY SITE AND SPONGE SAMPLING**

Sponge samples (n = 40) of the two target, common Mediterranean species – *Chondrosia reniformis* (HMA) and *Spirastrella cunctatrix* (LMA), were collected in July 2021 by scuba diving at 2-4 m depth in individual zip bags. Sponge species taxonomical confirmation was based on their morphological features and DNA barcoding (results not shown). Environmental seawater was collected in triplicate (5 L, n = 3) per site near the sponge assemblages. To minimize variability, two sites with similar environmental parameters (e.g., depth, temperature, orography) were selected. Sampling sites (Figure 2.4) were located around Ischia Island in the Gulf of Naples (Italy) and consisted of two semi-submerged caves: the acidified cave Grotta del Mago (40°42ʹ41.87ʹʹN, 13°57ʹ51.06ʹʹE; hereafter ‘vent’) presenting CO₂ bubbling and pH values of 7.6 – 7.88; and an control cave Punta Vico (40°45ʹ32.28ʹʹN, 13°52ʹ55.38ʹʹE; hereafter ‘control’) with pH values of 8.04 – 8.05 (Teixidó et al., 2020). After sampling, sponge individuals were transported in coolers to the laboratory, where subsamples (~0.5 cm³) were rinsed with sterile seawater, snap-frozen in liquid nitrogen and stored at -80°C until analyses. Seawater samples (1 L) were filtered through 0.22 μm pore size filters (Millipore MF™-Membrane), and filters were stored at -80 °C until processing.

Co-variates and the corresponding sample group codes are summarized in Table 2.1, which are used throughout downstream analyses, where the first letter indicates the initial letter of the corresponding genus for each sponge species, and the second letter the site code: CV and CM referring to *C. reniformis* from the control site ‘Vico’ and vent site ‘Mago’; SV and SM *S. cunctatrix* from the corresponding sites, and SWV and SWM representing seawater from the two study sites.
Figure 2.4: Location of the study site Ischia Island (A) and the acidified and control semi-submersed caves, Grotta del Mago and Punta Vico, respectively. Cave entrances of Control site Punta Vico (blue; B) and CO$_2$ vent site Grotta del Mago (red; C). Photography by Jana Efremova.

Table 2.2: Sample Codes. Codes were made up by combination of letters, where C and S represented the target species C. reniformis, S. cunctatrix and SW the seawater; and V and M represented the site code for Grotta del Mago vent site and Grotta Punta Vico control site respectively.

<table>
<thead>
<tr>
<th>Species</th>
<th>Abbreviation</th>
<th>Control ('Vico')</th>
<th>Vent ('Mago')</th>
<th>n</th>
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<tbody>
<tr>
<td><em>Chondrosia reniformis</em>  (HMA)</td>
<td>CV</td>
<td>x</td>
<td></td>
<td>10</td>
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<tr>
<td></td>
<td>CM</td>
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<td>x</td>
<td>10</td>
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<tr>
<td><em>Spirastrella cunctatrix</em> (LMA)</td>
<td>SV</td>
<td>x</td>
<td></td>
<td>10</td>
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<tr>
<td></td>
<td>SM</td>
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<td>Seawater</td>
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**DNA EXTRACTION, LIBRARY PREPARATION AND SEQUENCING**

DNA from frozen sponge samples and seawater filters (-80°C) was extracted using QIAGEN PowerSoil Pro Kit (2018), following manufacturer’s instructions. Quantification and quality control of the extracted DNA was assessed through a Thermo Scientific NanodropTM 1000. Aliquots of the extracted DNA were sent to the Station Biologique de Roscoff (Platform Genomer; SBR France) for amplicon library preparations and sequencing. This research has been supported by EuroMarine and EMBRC.

The amplification of the 16S-v3-v4 specific locus (*Escherichia coli* position: 341-805) was performed following the 16S Metagenomic Sequencing Library Preparation protocol (Illumina Inc., 2013) for Illumina MiSeq Systems. The following bacterial and archaeal universal primer pairs were used: forward primer Bakt_341F 5'TCGTCGGCAGCGTCAGATGTATAAGAGACAG-[CCTACGGGNGGCWGCAG]3’ and reverse primer Bakt_805R 5’GTCTCGTGGGCTCGAGATGTATAAGAGACAG-[GACTACHVGGGTATCTAATCC] 3’ (Fadeev et al., 2021; Wasimuddin et al., 2020).

PCR Amplification reaction mix was prepared using: Metagenomic DNA (5 ng/μL) 2.5 μL, forward primer (1 μM) 5 μL, reverse primer (1 μM) 5 μL, KAPA HiFi HotStart ReadyMix 12.5 μL, for a total volume of reaction of 25 μL. PCR were performed in a 96-Well Thermal Cycler following this thermocycling conditions: 95°C for 3 minutes followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds and a final elongation performed at 72°C for 5 minutes and a 4°C hold. Before sequencing, PCR products were purified with the AMPure XP beads to eliminate free primers and primer dimer species. Sequencing was performed on a MiSeq sequencer (Illumina platform) with a Reagent Kit v3 (600-cycle) targeting ~400 – 450 bp amplification products. Raw sequences will be available upon publication on [https://www.ncbi.nlm.nih.gov/sra/PRJNA926125](https://www.ncbi.nlm.nih.gov/sra/PRJNA926125).

**BIOINFORMATIC WORKFLOW**

The bioinformatic workflow used to analyse sequencing output data is depicted in Figure 2.5. Various tools were used to achieve a detailed characterization of the sponge microbiomes and seawater microbial communities. Aim was to compare HMA and LMA sponge microbiomes and to identify shifts in their microbial communities between the CO₂ vent and control site. The unrarefied feature table was used to create Venn Diagrams and perform core microbiome analyses, as well as compositional data analyses. Compositional methods included the estimation of compositional beta diversity using DEICODE ([https://github.com/biocore/DEICODE](https://github.com/biocore/DEICODE)) and the identification of differentially abundant features using the tools Gneiss ([https://github.com/biocore/gneiss](https://github.com/biocore/gneiss)), Songbird ([https://github.com/biocore/songbird](https://github.com/biocore/songbird)) and DeSeq2 (Love et al., 2014). Rarefied
abundance tables were used to compare traditional diversity metrics, including alpha and beta diversity measures and stacked bar plots of relative abundances.

Figure 2.5: Bioinformatic workflow for microbiome community analysis.

**PROCESSING OF SEQUENCING DATA AND QUALITY CONTROL**

A total of 49 samples, including blanks and PCR negatives were sequenced, and the resulting demultiplexed fastq files were imported into QIIME2 v.2021.11 (Bolyen et al., 2018), for sequence processing, quality control filtering and downstream analyses. Raw sequencing reads, with a ranging length between 280 and 444 bp, were first corrected for sequencing errors by quality filtering and the construction of Amplicon Sequencing Variants (ASVs) using the QIIME2 plugin DADA2 (Callahan et al., 2016). Sequences were visualized in an interactive quality plot to decide for optimal truncation option, where Phred quality values were accepted at >20. Amplicon primers were removed and high-quality sequences were obtained by truncating at nucleotide positions 280 and 210 bp for left and right truncation, respectively. Following denoising steps filtered out noisy sequences, removed chimeric sequences and singletons and joined denoised paired-end reads. The resulting sequences were dereplicated to combine all identical sequencing reads into ‘unique sequences’ with a corresponding ‘abundance’ equal to the number of reads with that unique sequence.

**TAXONOMIC ANNOTATION**

Taxonomy was assigned against the 16S SILVA v.138_1 reference database (Quast et al., 2013), which was pre-trained using the qiime2 RESCRIPt pipeline, and evaluated with the evaluate-fit-classifier command (Robeson et al., 2021). RESCRIPt 584 (REference Sequence annotation and CuRation Pipeline) is a python package and qiime2 plugin for formatting, managing, and manipulating sequence reference databases. This package was used to compile, manipulate, and evaluate sequences from the 16S SILVA v.138_1 reference database (with 99% identity) (Quast et al., 2013) and to finally construct an optimized reference database for taxonomical annotation in
QIIME2. Reference reads were trimmed to span the 16S gene v3-v4 region (i.e., 341f - 785r = 444 bp). Quality control of taxonomical annotation revealed high precision until level 6 (genus), supported by the integrated volatility control chart. The volatility plot showed for each taxonomical level (1 = phylum until 7 = species), corresponding precision and relevance of taxonomical annotation compared to reference results (‘F-measure’). Global mean values for annotation precision determine the control limits, which are ± 2× and 3× standard deviation from global mean. Observations above or below those global control limits are suspected to be outliers (Estaki et al., 2020). Curve progression suggested, that precision of taxonomical annotation at level 7 (species) dropped under the lower control limit.

The resulting ASV feature table was then filtered considering a minimum of 10 reads counted throughout all the samples. All features whose taxonomy contained untargeted assignments were removed, such as chloroplast and mitochondria. After these pre-processing steps, the final unrearified ASV table was obtained and used for data analyses. Alpha-rarefaction plots were generated using the alpha rarefaction tool (q2-diversity) to visualize rarefaction curves and reveal the lowest number of reads, which were sufficient to reach a plateau in which diversity would not increase with deeper sequencing (Figure S.1). This guaranteed a full coverage of bacterial/archaeal diversity, while retaining the maximum number of samples for the analysis.

A rarefied feature table for standard diversity calculations was obtained by rarefying the ASV tables at a mean sequencing depth of 1,173 reads. This allowed to standardize the library size across samples, mitigating challenges due to biases in original measurements (Weiss et al. 2017). All samples resulted in total counts above the defined threshold and hence were retained. After rarefaction, the dataset included 390 different ASVs, distributed in 46 samples, with a frequency ranging 1,173 – 46,909 per sample. The resulting abundance tables of annotated ASVs were then imported into R v. 4.2.0 for downstream analyses.

MICROBIAL DIVERSITY

To explore bacterial diversity across sponge samples and seawater, alpha diversity (within samples) and beta diversity (between samples) were computed applying core-diversity-phylogenetics in QIIME2. This command provided the alpha diversity measures observed features, Shannon diversity, Pielou’s evenness and Faith’s phylogenetic diversity. Beta diversity metrics were estimated using distance matrices based on (un)weighted UniFrac distances, Bray-Curtis and Jaccard indices. Since alpha and beta diversity calculations are sensitive to uneven sampling depths, rarefied feature tables were used as input. Rarefaction allowed to adjust for differences in library sizes across samples and allow comparisons between samples. Alpha diversity analysis were represented as boxplots using ggplot2 (Wickham, 2016). Statistical testing was computed on QIIME2 using pairwise Kruskal-Wallis-Tests to reveal differences between
sample groups (p < 0.05) and pairwise Kruskal–Wallis tests with Benjamin–Hochberg false discovery rate (FDR) corrections for multiple comparisons (q < 0.05; i.e., FDR adjusted p value).

For beta diversity analyses, community distance matrix based on (un)weighted UniFrac distances, as well as Bray-Curtis and Jaccard distances, were visualized with the qiime2 visualization tool ‘Emperor’ as PCoA plots. Permutational analysis of variance (PERMANOVA, Adonis) and pairwise analysis of similarity (Anosim) were computed in qiime2 to evaluate changes in microbiome structure and composition per species across sites. All significance thresholds for statistical tests were set at p < 0.05.

Variations in community structures were determined with beta dispersion analyses (permdisp) applied for all beta diversity distance matrices. The analyses of multivariate dispersion of beta diversity metrics, measures the average dissimilarity from individual observation units (here: ASVs) to their group centroid in a multivariate space, using an appropriate dissimilarity measure (Anderson et al., 2006). Permdisp tests were set to 999 permutations and significance to q < 0.05 (i.e., false discovery rate adjusted p value).

Relative abundances were computed based on rarefied data, and were visualized in stacked bar plots grouped per metadata categories, i.e. species and site using ggplot2 (Wickham, 2016). The Qiime2R package was used to import qiime2 artifacts into the R environment as R objects (https://github.com/jbisanz/qiime2R). Further packages used for downstream analyses and plotting were phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen et al., 2008).

**CORE MICROBIOME ANALYSES**

Venn diagrams of total species richness in both environments were generated using the eulerr package (Larsson, 2022). Core microbiome analyses were performed on unrarefied data, and core ASVs were defined at 99% of prevalence. Bullseye plots for the representation of core sizes and donut plots for the core microbiome composition were produced with ggplot2 (Wickham, 2016).

**COMPOSITIONAL DATA ANALYSES**

Microbial diversity studies using classical methods (α and β diversity, taxa bar-plots) are widely used, but are largely driven by fluctuations among high-abundance taxa in a microbial community, while rare species are easily overseen (Martino et al., 2019; Morton et al., 2019; Willis, 2019). Furthermore, interpretations of these results can be misleading, because microbiome data are of compositional nature with high levels of sparsity. The lack of knowledge about original microbial loads introduce further bias (Gloor et al., 2017). Rarefaction of these data as a practice of normalization is inappropriate for the detection of differentially abundant taxa, as suggested by McMurdie and Holmes (2014). One solution to this problem is to complement diversity estimations with compositional data analyses techniques.
Figure 2.6: Schematic representation of differentials as log fold ratios of ASV abundances used in compositional data analyses. The size of each cube represents the abundance of a defined feature (ASV) in samples from vent (red: 'Vent') versus control (blue; 'Ctrl') site. The subsequent log transformation of the ratio results in the log2fold change and a positive or negative correlation to defined category (here: vent). Visualization adapted from Morton et al. (2017).

Compositional data analyses, which aims at identifying differentially abundant taxa compares ratios of each feature across defined metadata categories and cancels out the before mentioned biases and the need of knowing the initial microbial load. By determining the logarithmic ratios of microbial balances (Figure 2.6), compositional data analyses give equal weights to relative increases or decreases of each microbial feature, regardless of their relative abundance. Several compositional data analyses tools were applied for this thesis chapter to describe compositional beta diversities (DEICODE), visualize differentials in heatmaps (gneiss) and identify differentially abundant features, which likely drove microbial community changes in *S. cunctatrix* and *C. reniformis* in response to OA.

**DEICODE: COMPOSITIONAL BETA DIVERSITY**

Compositional beta diversity analyses were based on Aitchison distances using the qiime2 plugin DEICODE (https://github.com/biocore/DEICODE), a pipeline that works on non-rarefied data applying matrix completion and robust principal-component analysis (RPCA). DEICODE is robust to high levels of sparsity (Martino et al., 2019) by solving the ‘zero-problem’ applying matrix completion (Keshavan et al., 2009). The distance matrix was calculated with a non-supervised method (no co-variates were introduced in a formula). DEICODE revealed ASVs which were responsible for beta-diversity shifts and visualized these niche features as factors in compositional RPCA (Robust Principal Component Analysis) biplots. Biplots were first inspected using the qiime2 integrated visualization tool Emperor. Final biplots of DEICODE ordinations were produced in R using ggplot2 (Wickham, 2016).

Log ratios of selected features driving the differences in the ordination spaces were then visualized with Qurro (Fedarko et al., 2020) (https://github.com/biocore/qurro). Statistical differences of the
resulting log ratios between different sample groups, i.e. sponge species or seawater from vent versus control site, were determined using the Welch’s Heteroscedastic F Test (p < 0.05).

**Gneiss**

A multivariate response linear regression model was computed using the qiime2 plugin gneiss. Gneiss is a compositional data analysis and visualization tool based on the concept of balances with log ratios. This plugin investigates relationships between proportions without the limitations and biases that come along with dependency properties between ASVs. Balances are computed by creating a log-transformation of abundance ratios:

$$\log = \frac{\text{abundance of numerator ASV}}{\text{abundance of denominator ASV}}$$

Principal balances were obtained by unsupervised hierarchical clustering of ASVs (Morton et al., 2017; Ward, 1963). Then mean log abundances were compared between all subtrees and branches down to ASV levels. Very rare ASVs with less than 10 reads across all samples were removed from the unrarefied feature table before correlation clustering. Differentially abundant ASVs across different sites were visualized in a heatmap. Since this tool gave only information about the presence/absence of differentials, I complemented the analysis, by identifying differentially abundant ASVs with bioinformatic tools described in the following sections.

**Songbird**

Songbird (v1.0.3) was used to identify differentially abundant features (ASVs), by determining the log-fold changes of ASVs associated to *C. reniformis* and *S. cunctatrix* from control and vent sites (Morton et al., 2019). The main output produced by Songbird is a file, containing differentials, i.e. log-fold changes of features (here: ASVs) with respect to sample metadata (here: site). Importantly, these features are ranked according to their association with the given covariate. Subsets of unrarefied feature tables were analysed separately for each sponge species to eliminate ‘species’ as a covariate and focus solely on the association to ‘vent site’.

Briefly, Songbird uses a multinomial regression model to calculate differential ranks. Multinomial regression were implemented using TensorFlow (Abadi et al., 2016) according bioinformatic recommendations provided at https://github.com/biocore/songbird. Two models were created separately for *C. reniformis* and *S. cunctatrix*. For model generation, the formula ‘site’ specified the covariate to be included in the model. Optimal parameters were determined by checking the fit of the model to avoid overfitting. Overfitting and the accuracy of each generated model was determined, by comparing them to a null model. A null model was generated by supplying the initial formula with ‘1’. A resulting pseudo $Q^2$ value close to 1 indicated a high predictive accuracy on the cross-validation samples. A pseudo $Q^2$ value very close or below zero, represented poor predictive accuracy, suggesting possible overfitting.
Differential ranks were confirmed by generating log-ratios and statistical boxplots through ‘Qurro’ (Fedarko et al., 2020) following the tutorial found at https://github.com/biocore/qurro. The produced sample boxplots included log fold changes of selected features and grouped their differences across sample groups. Statistical differences of the log ratios were determined using the Welch's Heteroscedastic F Test ($p < 0.05$).

**DESeq2**

DESeq2 (v 1.36.0) was additionally applied to confirm identified differentially abundant ASVs. DESeq2 is a method for differential analysis of count data, originally developed to estimate fold changes of RNAseq data, but can also be applied to other high-throughput sequencing count data (Love et al., 2014). This tool aims at integrating several methodological approaches of differential abundance analyses, including the feature ranking by fold changes and introduces novel features to perform quantitative analyses using shrinkage estimators for dispersion and fold change. The advantages of this method include a highly sensitive and precise feature ranking, including a customizable visualization tool. It further controls false positive rates. The variability between replicates is modelled by a dispersion parameter. The accurate estimation of a dispersion parameter is critical for statistical inferences of differential expression (Love et al., 2014).

To generate a model, DESeq2 fits a generalized linear model (GLM) (McCullagh and Nelder, 1989) with a logarithmic link to each feature, following a negative binomial distribution. In case of a comparison between two groups, such as ‘treated’ versus ‘control’ (in our case: vent site versus control site), the matrix elements indicate whether a sample is ‘treated’ or not, while the GLM fit returns coefficients indicating the overall expression strength of each feature and the log2fold change between vent and control site.

The DESeq2 package was used in R as a Bioconductor package and was obtained from http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html. Differentials were computed, defining the log fold change $\geq 2\mid$ (Benjamini–Hochberg (BH)-adjusted p-value $< 0.05$) through pairwise comparison between vent and Control site. Only ASVs with $> 10$ counts were included in the analysis.
**QURRO**

The tool for Quantitative Rank/Ratio Observations ‘Qurro’ (Fedarko et al., 2020) ([https://github.com/biocore/qurro](https://github.com/biocore/qurro)) was used to visualize and explore the estimated log-fold changes for feature differentials and their rankings, which resulted from Songbird (differentials) and DEICODE (feature loadings) analyses. Qurro produces an interactive feature ranking plot and a sample plot to examine particularly high- or low-ranked features in our dataset. The feature ranking sorted features according to their log-fold change with respect to how they were correlated to a metadata co-variate category, e.g., how distinctly the log-ratios differed between sponge samples from vent versus control sites.

From here microbial balances were created to separate sample groups, with numerator taxa being positively associated with a certain category (in our case: vent), and denominator taxa negatively ranked to this category, and vice versa. The microbial balances yielded log-ratio values that were represented as boxplots, and were statistically tested for differences among sample groups. Higher log-ratios in a sample group were correlated to numerator features and negatively ranked to denominator features (e.g. if samples from vent site had higher log-ratios, then these samples were associated to an increased abundance of the numerator taxa, or decrease in denominator taxa); while lower log-ratios are ranked to denominator features and negatively correlated to numerator taxa (e.g. if samples from control site had lower log-ratios, then these were correlated to denominator features). Differences in log-ratios resulting from selected numerator and denominator taxa were statistically evaluated in R, using the Welch's Heteroscedastic F Test ($p < 0.05$).

**SEAWATER ANALYSES**

**INORGANIC AND ORGANIC NUTRIENTS**

For inorganic nutrient analyses seawater was filtered through cellulose acetate membrane filters (pore size: 0.22 μm) into 20 mL high-density polyethylene vials and stored at -20°C until analysis. Measurements quantified dissolved inorganic nitrogen (DIN: ammonium, nitrate and nitrite) and dissolved inorganic phosphate and silicate. Analyses were carried out on a five-channel continuous flow autoanalyzer (Flow-Sys Systea), according to Hansen and Grasshoff (1983).

For total organic carbon (TOC) and nitrogen (TN), samples were collected into acid pre-washed 30 mL HDPE sample bottles. Before the measurements, samples were acidified with HCl 37% ($pH \leq 2$) to remove inorganic carbon. The analyses were carried out by using a Shimadzu TOC-L analyzer. The reliability of the measurements of organic carbon was verified daily by comparison with Consensus Reference Waters (Hansell, 2005; Sharp et al., 2002). Potassium phthalate was used as
standards. TN was measured simultaneously with TOC by using the TN unit connected to the Shimadzu TOC-L analyzer. Potassium nitrate was used as standard, whereas the seawater reference material from the Consensus Reference Material Project (CRM; http://yyy.rsmas.miami.edu/groups/biogeochem/CRM.html) was used to determine the precision and accuracy of the method. All nutrient measurements were performed at the Stazione Zoologica Anton Dohrn, Italy.

**Quantification of planktonic cells (FACS)**

The quantification of picoplanktonic cells in the water column (autotrophic and heterotrophic, both prokaryotes and picoeukaryotes) were determined using flow cytometry for seawater samples (triplicates) of both sites. Sample processing required 1 mL aliquots of seawater samples, which were fixed with a mix of paraformaldehyde and glutaraldehyde (1% and 0.01% final concentration, respectively) and directly snap-frozen in dry ice or liquid nitrogen. Upon arrival to the laboratory, samples were stored at -80°C until analyses (Marie et al., 1999). Cytogram analysis was conducted using FCS Express software (De Novo Software, Los Angeles, CA). Cytometric clusters included *Synechococcus* spp. and picoeukaryotes, discriminated based on their relative scatter and autofluorescence (orange from phycoerythrin and red from chlorophyll for the first, and red only for the latter). Heterotrophic prokaryotes, which are not autofluorescent, were discriminated from background noise based on their scatter and green fluorescence (from SYBRGreen stain, proxy of DNA content), as in Marie et al., 1999 and Balestra et al., 2011. The flow cytometer used is a Becton Dickinson FACSVerse instrument with standard filter set.

**Light and temperature measurements**

Seawater temperature and light intensity at the two study sites were recorded hourly from year 2017 until 2021 for temperature and 2020 until 2021 for light irradiation, using Water Temperature Data Loggers (HOBO TidbiT v2, Onset) and Odyssey Integrating Photosynthetically Active Radiation (PAR) Sensor (Dataflow Systems PTY Limited), respectively. All sensors were deployed in the caves around 5 meters distance from the sponge assemblages, at 3 m depth. The Odyssey PAR sensors were previously calibrated with a LICOR PAR sensor (LI-1400). Irradiance was measured as µmol photons m-2 s-1 and expressed as the log10 for better visualization.
RESULTS

SPONGE MORPHOLOGY AT VENT AND CONTROL SITES

Morphological changes were observed for *S. cunctatrix* at the vent site. The LMA sponge (Figure 2.7) showed reduced body surface area, narrower water canals and smaller oscula with superficial signs of tissue necrosis (Figure 2.7B). Diameter of oscula ranged in control conditions between 3 and 5 mm, whereas at the vent site maximum oscula size was ≤ 1 mm. The HMA sponge (Figure 2.8) instead, had similar body volume and oscula sizes across both sites.

Figure 2.7: LMA sponge *S. cunctatrix* from control (A) and vent site (B). Photography by Jana Efremova.

Figure 2.8: HMA sponge *C. reniformis* from control (A) and vent site (B). Photography by Jana Efremova.
STUDY SITE PARAMETERS

The two study sites, the CO$_2$ vent system and the control site showed similar conditions for light (Figure 2.9) and temperature (Figure 2.10), as well as similar nutrient availabilities (Figure 2.11, Figure 2.12). Increased levels of planktonic communities were measured at the CO$_2$ vent site (Figure 2.13).

LIGHT DATA

Photosynthetically active radiation (PAR) revealed similar light conditions for the summer months across July and beginning of August, when sponge and seawater sampling was conducted. Between middle of August and October, light conditions were lower at the control site compared to the CO$_2$ vent site (Figure 2.9).

Figure 2.9: Mean daily irradiance (PAR in µmol s$^{-1}$ m$^{-2}$) of control pH and CO$_2$ vent site.
**TEMPERATURE**

Temperature was similar across both sites and followed seasonal fluctuations (Figure 2.10), ranging from 14.7 to 15.2 °C in winter (n = 16,754) and from 25.6 to 26.5 °C in summer (n = 19,011).

![Figure 2.10: Average temperature (°C) of the study sites from 2017 until 2021.](image)

**NUTRIENTS**

Inorganic nutrients showed no significant differences between control and vent sites in the years 2018, 2019 and 2021 (Figure 2.11). Nutrient data for the years 2018 and 2019 were obtained from Teixidó et al. (2020). In 2021, silicate concentrations of 1.72 ± 0.07 mmol m⁻³ at the control site showed a decrease at the vent site down to 0.76 ± 0.02 mmol m⁻³. However, this difference could not be observed in 2019 or 2018. Similarly, nitrate concentrations dropped from 1.45 ± 0.01 mmol m⁻³ down to 0.35 ± 0.01 mmol m⁻³ at the vent site only in 2021. Phosphates, ammonium and nitrite were similar across sites.
Inorganic nutrient concentrations for silicates, phosphates, ammonium, nitrate and nitrite for the control site and the CO$_2$ vent site. Shown are mean concentrations in mmol m$^{-3}$ ± standard deviation. *data from Teixidó et al. (2020).

Total organic carbon (TOC; Figure 2.12A) and total organic nitrogen levels (TN; Figure 2.12B) were similar across the CO$_2$ vent site and the control site. Measured TOC levels revealed 160.58 ± 61.86 µM for the CO$_2$ vent and 161.59 ± 68.41 µM for the control site. Final TN concentrations showed no differences between the CO$_2$ vent (11.53 ± 1.62 µM) and the control site (11.63 ± 2.06 µM).

Typical ultra-planktonic prey taxa of sponges, such as *Synechococcus* and picoeukaryotes, showed a 2.5- and 3-fold increase at the vent site, respectively (Figure 2.13). Seawater samples collected at the control site had 20 921 ± 802 *Synechococcus* and 549 ± 150 picoeukaryote cells per mL seawater, while at the CO$_2$ vent site cell counts recorded up to 51 909 ± 1 299 and 1 753 ± 210 cells per mL, respectively.
Figure 2.13: Pico-plankton values for *Synechococcus* and Picoeukaryotes obtained from FACS analyses of seawater samples. Shown are mean concentrations in cells/mL ± standard deviation.

16S rRNA SEQUENCING OUTLOOK

The sequencing of the 16S v3-v4 region yielded a total of 346,123 reads and 1,676 ASVs across 46 samples after pre-processing steps for high quality sequences (ranging from 1,350 to 48,948 reads per sample). The rarefaction of this feature table at mean sampling depth of 1,173 yielded 319,408 reads and 390 ASVs (Table 2.2). Total raw reads per sample type and site are summarized in Table 2.3, including reads maintained after each pre-processing step, including filtering, denoising, merging and the removal of chimera (PCR errors), mitochondria and chloroplast. Sequencing raw data are available on [https://www.ncbi.nlm.nih.gov/sra/PRJNA926125](https://www.ncbi.nlm.nih.gov/sra/PRJNA926125) after the release date (31.05.2023) or publication of according data in a paper.

Table 2.3: Sequencing results for whole dataset, including rarefied and non-rarefied feature tables.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Rarefaction depth</th>
<th>Number ASVs</th>
<th>Total frequency</th>
<th>Median frequency per sample</th>
<th>Min. frequency per feature</th>
<th>Max. frequency per feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unrarefied</td>
<td>46</td>
<td>-</td>
<td>390</td>
<td>319 408</td>
<td>4 473</td>
<td>10</td>
<td>26 240</td>
</tr>
<tr>
<td>Rarefied</td>
<td>46</td>
<td>1173</td>
<td>385</td>
<td>53 958</td>
<td>1173</td>
<td>10</td>
<td>7 348</td>
</tr>
</tbody>
</table>

Table 2.3: Total Reads per pre-processing step.

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<tr>
<th></th>
<th>Raw</th>
<th>Filtered</th>
<th>Deinoised</th>
<th>Merged</th>
<th>Non-chimeric</th>
<th>% maintained</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Seawater</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mago</td>
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<td>28820</td>
<td>23452</td>
<td>15434</td>
<td>15434</td>
<td>16%</td>
</tr>
<tr>
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<td>17714</td>
<td>14291</td>
<td>9742</td>
<td>9742</td>
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</tr>
<tr>
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<td>25176</td>
<td>25176</td>
<td>18%</td>
</tr>
<tr>
<td><strong>C. reniformis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mago</td>
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<td>149152</td>
<td>140593</td>
<td>119714</td>
<td>100963</td>
<td>21%</td>
</tr>
<tr>
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<td>207491</td>
<td>190155</td>
<td>153752</td>
<td>129550</td>
<td>17%</td>
</tr>
<tr>
<td>Total</td>
<td>1264387</td>
<td>356643</td>
<td>330748</td>
<td>273466</td>
<td>230513</td>
<td>18%</td>
</tr>
<tr>
<td><strong>S. cunctatrix</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>53481</td>
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<td>567852</td>
<td>507699</td>
<td>400952</td>
<td>346123</td>
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</table>
MICROBIOME DIVERSITY

ALPHA DIVERSITY

The HMA sponge *C. reniformis* showed generally higher alpha diversity than the LMA sponge *S. cunctatrix across all diversity metrics (Kruskal-Wallis, \( p < 0.001 \); Figure S.2). Both species showed significantly lower evenness and Shannon diversity than the seawater. The LMA sponge had significantly less observed features than seawater. Phylogenetically, the microbiome of the HMA sponge showed highest Faith’s diversity.

Alpha diversity metrics across CO\(_2\) vent and control sites revealed different trends in HMA sponges compared to LMA sponges and the seawater (Figure 2.14). Observed features and Shannon indices were significantly lower for *C. reniformis* in the vent site compared to control samples. No differences in observed ASVs were detected in *S. cunctatrix* between both sites, however a significantly higher Shannon index at the vent implies increased species richness and evenness. The microbiome of the HMA sponge samples had in both sites significantly higher phylogenetic diversity compared to its LMA counterpart. No differences were detected comparing vent to control conditions within either sponge species. *C. reniformis* had significantly higher Pielou’s evenness than *S. cunctatrix* in control conditions, but not at the vent site. While *S. cunctatrix* showed significantly higher evenness at the vent site, *C. reniformis* and seawater samples had no differences across sites. Seawater from the vent site had significantly more observed features, and an increased Shannon and Faith’s phylogenetical diversity relative to control site.

![Figure 2.14: Alpha diversity metrics for *C. reniformis*, *S. cunctatrix* and seawater from vent site and control site. Boxplots show range (whiskers), median (bold line), and interquartile range (box height).](image-url)
**Beta Diversity**

Differences in beta diversity were analysed using four beta diversity distances: unweighted and weighted UniFrac, Jaccard and the Bray-Curtiss dissimilarity (Figure 2.15). Both sponge species and seawater had significantly different microbial community compositions, according to all diversity metrics.

![Figure 2.15: PCoA of beta diversity metrics for C. reniformis, S. cunctatrix and seawater-associated bacterial communities from CO₂ vent and control site. Three-dimensional PCoA plots show the separation of sponge individuals and seawater samples and their separation across sites.](image)

Comparing samples from vent versus control sites, the Jaccard index (a qualitative measure of community dissimilarity) revealed best clustering, and significant differences were found for pairwise comparison of all sample groups (Pairwise Anosim; p < 0.01). Only seawater from the vent site compared to control site showed no statistical significance, despite forming separated clusters. Pairwise permdisp results revealed that within group variability of microbial communities were significantly different for *S. cunctatrix*, but not for *C. reniformis*. These results underline, that the differences in the microbiome of *C. reniformis*, were strongly driven by compositional differences between communities and not within-community variability. Instead for *S. cunctatrix*, the simultaneous significance in Anosim and Permdisp, suggest that despite the centroids of each microbial community being significantly different, increased within-community variability might confound this result. However, two clearly separated cluster were obtained also for *S. cunctatrix*,
supporting different beta diversity across sites. The Adonis Tests (Analysis of variance using distance matrices) revealed that Species, Acidification, as well as the Interaction between Species:Acidification showed significant impact on beta diversity based on Jaccard distances (Pr (>F) = 0.001).

The second best clustering was obtained using unweighted UniFrac distances, a qualitative beta diversity distance which takes into account phylogenetic diversity and emphasizes rare species (Lozupone et al., 2011). The PCoA plot resulted in 6 clusters and separated both sponge species from each other and from seawater, as well all samples from control and vent sites. However, some mixed sample projections from control and vent sponges confound the grouping for C. reniformis. Pairwise Anosim comparisons revealed that all clusters were significantly distant from each other (p < 0.01). Weighted UniFrac distances (emphasizes dominant species) separated sponge species and seawater from each other. Clustering depending on control and vent site were distinct for S. cunctatrix and seawater. C. reniformis samples were poorly separated and aggregated in one disperse cluster. Surprisingly, pairwise Anosim testing revealed that all groups were significantly different from each other (p < 0.01). Based on the Bray-Curtis dissimilarity (a quantitative measure of community dissimilarity) no visible clusters were obtained in the PCoA space for sponge and water samples from vent and control sites. However, pairwise Anosim results showed that all sample groups were statistically different from each other. Beta dispersion testing on (un)weighted UniFrac and Bray-Curtis distances, revealed significant differences in within-group variability in S. cunctatrix microbiomes across sites, but not in C. reniformis, confirming results obtained using Jaccard and reflecting a generally high microbial variability (i.e. instability) between LMA sponge individuals. Comparing seawater samples across control and vent site, no significantly different community composition was found according to any of the presented distance metrics.

**RELATIVE ABUNDANCE SHIFTS OF TAXA IN DEPENDENCE OF OCEAN ACIDIFICATION**

Relative abundances of the dominant bacterial taxa displayed shifts across control and vent sites from the phylum down to the genus level in sponges and seawater samples (Figure 2.16). The dominant phylum in sponges and seawater samples was Proteobacteria. In C. reniformis, Proteobacteria increased from 43±20% to 61±16% at the vent site, mainly due to an increase of Gammaproteobacteria (from 30±16% to 46±12%). The following most abundant phyla in C. reniformis were Chloroflexi, Poribacteria and Acidobacteria, which decreased their relative abundances at the vent. Chloroflexi decreased from 19±10% to 11±5%, driven by decreases of the class Dehalococcoidia with its clade SAR202 and the class Poribacteria. SAR202 decreased by approximately 50% from relative abundances of 12±7% at the control site to 6±5% at the vent site.
Poribacteria decreased from 12±8% to 5±7% at the vent site. Decreases of the phylum Acidobacteria from 11±5% to 5±5% were due to the classes Thermoanaerobacilia (4±2% to 1±1%) and Vicinamibacteria (4±2% to 1±2%). The gammaproteobacterial order Pseudomonadales notably increased at the vent site from 9±7% to 22±11%. At the genus level, this order was dominated by *Endozoicomonas*. *Candidatus Kaiserbacterium* increased at the vent site from 3±3% to 7±7%.

*Burkholderiales* (8±8%) and *Bdellovibironales* (4±2%) did not change their relative abundances between both sites, while the genus *Bdellovibrio* increased from 3±2% to 5±2% at the vent site.

**Figure 2.16**: Taxa bar plots showing microbial relative abundances for *C. reniformis*, *S. cunctatrix* and seawater from the vent and control site. Taxonomical annotation is presented for the top 15 taxonomical levels at phylum (A), class (B), order (C) and genus (D) level.

The microbiome of the LMA sponge *S. cunctatrix* was largely dominated by Alpha- and Gammaproteobacteria. Proteobacteria decreased slightly from 83±6% to 71±6% at the vent site. The classes Alpha- and Gammaproteobacteria both decreased by 6%, i.e. from 47±3% to 41±8% and from 36±7% to 30±6%, respectively. Gammaproteobacterial shifts were mainly due to a decrease of genus UBA10353 from 29±7% down to 10±5%. Interestingly, shifts reported in the *S. cunctatrix*
microbiome were similarly reflexed in the microbial communities of the environmental seawater. These included, Proteobacteria decreases in the seawater from 65±4% to 44±3% at the vent site, mainly driven by Alphaproteobacteria, decreasing from 56±5% to 32±2%. Compared to the LMA sponge, Gammaproteobacteria were generally less abundant in the seawater and increased slightly from 9±2% to 12±2% at the vent site. Strong increases were identified for phylum Bacteroidota in both, *S. cunctatrix* (from 2±1% to 11±5%) and seawater (from 15±2% to 32±0%) at the vent site. Abundances for Actinobacteriota increased from 2±2% to 4±3% in *S. cunctatrix* and 1±1% to 8±0% in seawater communities. Cyanobacterial levels were stable across both sites, but were higher in seawater samples (17±1%) with respect to *S. cunctatrix* (5±2%). The alphaproteobacterial order SAR11, dominated by Clade II and Clade Ia, was present at 5±3% in control conditions, but <1% in the vent for *S. cunctatrix*. Flavobacterales increased notably at order level from 1±1% to 7±4% due to an increase of the genus *Zeaxanthinibacter*. The bacterial order Pseudomonadales (6±3%) remained stable across both sites.

In the surrounding seawater, the most abundant order SAR11 decreased under OA from 28±1% to 11±2%. Additionally, Puniceispirillales and Rhodospirillales decreased at the vent site from 9±1% to 3±2% and 6±0% to 3±1%, respectively. Analogous to the LMA sponge, a strong increase of Flavobacterales under acidified conditions was observed in seawater samples, recording a relative abundance of 23±1% at the vent site. This was a two-fold increase compared to the relative abundances at the control site (12±2%). The most dominant genus within Flavobacterales were the marine groups NS4, NS5 and uncultured microbes belonging to the family Cryomorphaceae. Additionally, the order Balneolales (control: 3±1% and vent: 6±1%) and Enterobacterales (control: 1±0% and vent: 5±2%) increased slightly in seawater samples from the vent site. Micrococcales was not present in control seawater, but showed a relative abundance of 7±1% at the vent. Generally, variability of relative abundances across sites was lower in seawater samples compared to sponge samples.

**Shared microbes among the LMA and HMA sponge and ambient seawater**

Venn diagrams were computed with unrarefied feature tables to represent the number of taxa that were shared between HMA and LMA sponges and the surrounding seawater in vent and control conditions (Figure 2.17). The total number of microbial taxa decreased at the vent site for both, the HMA and the LMA sponge, but increased for seawater samples. At the control site a total of 158 and 142 ASVs were identified in *C. reniformis* and *S. cunctatrix*, respectively. The number of ASVs decreased in acidified conditions to 127 and 106 taxa, respectively. Microbial taxa in seawater showed the opposite trend with an increase from 58 ASVs in control conditions to 85 ASVs at the
The HMA sponge from control and vent site shared 109 ASVs, however 18 and 49 ASVs were exclusive to vent and control samples, respectively. LMA sponges from vent and control sites only shared 58 ASVs and were characterized by a large proportion of unique ASVs – 48 in vent and 84 in control sponges. This underlines that a large proportion of ASVs were unique across vent and control holobionts belonging to the same species, suggesting symbiont switching.

HMA and LMA sponges had 35 overlapping features at the control site, mainly belonging to the classes Alphaproteobacteria (58%), Bacteroidia (20%) and Gammaproteobacteria (11%). At the vent site, shared ASVs dropped by 90% down to 4 ASVs, belonging to Alphaproteobacteria, Gammaproteobacteria, Verrucomicrobiae and Planctomycetes.

Figure 2.17: Venn Diagram of shared microbial taxa between the HMA, LMA sponge and seawater from control pH (left) and CO₂ vent site (right). * Shared core microbes, i.e. ASVs present in all 10 sponge replicates per site. This analysis was based on non-rarefied feature tables.

In control conditions, *S. cunctatrix* shared 30 ASVs (21%) with control ambient water, whereas in the CO₂ vent site, *S. cunctatrix* shared 20 ASVs (19%) with the seawater. In both sites, shared ASVs
between the LMA sponge and seawater were Alphaproteobacteria, Bacteroidia, Cyanobacteria and Gammaproteobacteria (listed in decreasing relative abundances). *C. reniformis* did not share any microbes with the seawater in the control site and only 1 ASV in acidified conditions, belonging to the phylum Verrucomicrobiota and genus *Haloferula*. The number of core ASVs in the HMA sponges was higher than in the LMA species (Figure 2.17; number of core taxa indicated with *). A decrease in the number of core taxa was observed in HMA sponges under OA, recording 17 core taxa in the control site versus 12 in the CO₂ vent site. Contrasting results were found for the LMA sponge, which had a total of 4 core taxa at the control site, and 9 core taxa at the vent site.

**CORE MICROBIOME**

The dynamics of core microbial communities in the HMA and LMA sponge species were studied to distinguish stable from transient bacterial groups under elevated CO₂ conditions. Core bacterial communities were strictly defined as features that were consistently present across all sponge replicates within each sample grouping (species, sites). The core microbiome size, was generally smaller for *C. reniformis* (50%) than for *S. cunctatrix* (68%), independently from sampling site (Figure 2.19A and B). When including both sampling sites into core microbiome analyses, i.e. determine the shared ASVs across all replicates (n = 20) per species, core sizes decreased in both species (Figure 2.19C and D). This effect was stronger in the HMA sponge *C. reniformis*.

The HMA sponge *C. reniformis* showed generally a more diverse core microbiome in both sites (Figure 2.19A). Two new phyla were introduced into the core microbiome at the vent site: Dadabacteria (order Dadabacteriales; 2±1%) and Nitrospirota (genus *Nitrospira*; 3±2%), while 4 phyla were lost: Poribacteria, PAUC34f, Acidobacteriota (order Vicinamibacterales) and Myxococcota. Several members of the core microbiota were rare taxa. The most prominent core taxa in control conditions were: Poribacteria (18±14%), Anaerolineae (i.e. an uncultured Choloflexus: A4b; 15±8%), *Endozoicomonas* (14±17%) and SAR202 (7±5%). The most dominant microbial phylum in the core microbiome of *C. reniformis* was Chloroflexi which comprised 35% of the core microbiome at the control site, but declined to 13% at the vent site. Proteobacteria were represented by 11% of Alphaproteobacteria and 16% of Gammaproteobacteria at the control site. At the vent site Gammaproteobacteria peaked to 55%, mostly due to *Endozoicomonas* comprising 34±16% and uncultured species. Similarly, the relative core abundance of Alphaproteobacteria increased at the vent site (from 11% to 22%), revealing a less diverse composition. At the control site Alphaproteobacteria were constituted evenly by *Albidovulum*, Puniceispirillales (EF100-94H03), Alphaproteobacteria (AT-s3-44) and uncultured species, whereas at the vent one uncultured Alphaproteobacteria feature made up 18±9% relative core abundance, and genus *Constrictibacter* (Puniceispirillales) was present at 2±1%.
Figure 2.19: Core size and shared core ASVs of *C. reniformis* and *S. cunctatrix*. Core microbiome analyses were performed with sponge samples (n=10) separately for the control or ambient site for both, *C. reniformis* (A) and *S. cunctatrix* (B). The shared core microbiome across sponge individuals from both acidified and control sites (n=20) are shown to identify stable and key core microbes for *C. reniformis* (C) and *S. cunctatrix* (D) in control and acidified conditions. Each upper bullseye plot represents the size of the core microbiome relative to the variable microbiome. Donut plots below show the ASV composition and relative abundances for each core microbiome with best hit taxonomical annotation.

The core microbiome of *S. cunctatrix* from the control site was largely dominated by Alpha- and Gammaproteobacteria (Figure 2.19B), comprising 48% and 50%, respectively. Gammaproteobacteria were represented by the dominant genus UBA10353 at 32±14% and one feature belonging to the family Thioglobaceae at 9±6% of relative core abundance. Interestingly, 6 new features were introduced into the core microbiome at the vent site, one belonging to genus
**Zeaxanthinibacter** (Bacteroidota; 10±6%), one **Cyanobium** (Cyanobacteria; 4±1%), one feature belonging to the family Thermoanaerobaculaceae (Acidobacteria; 2±1%), one uncultured Cyclobacteriaceae (6±3%), as well as two uncultured Alphaproteobacteria and one Gammaproteobacteria. Concomitantly, the relative abundance of core Gammaproteobacteria decreased by 20%, in particular UBA10353 decreased down to 13±8%.

Core size decreased remarkably in HMA sponge across sampling sites, revealing very few core taxa shared between the vent and control site (Figure 2.19C). There was a drop of mean core size when comparing C. reniformis individuals from one site (control or vent) to both sites (control + vent), with decreases from 50±11% down to 23±10% core size, respectively. Shared core ASVs of both sites in C. reniformis were dominated by **Endozoicomonas** (44±26%), followed by Anaerolineae (A4b; 27±19%), Dehalococcoidia (SAR202; 13±9%), **Bdellovibrio** (9±9%) and a terrestrial genus BD2-11 (Gemmatimonadota; 6±5%). The core size and composition of S. cunctatrix across both sites was similar to the core microbiome across individuals of only the control site (Figure 2.19D). When considering individuals from both sites, the core size dropped by 10%, recording an abundance of 54±16%. The 3 core ASVs included, one uncultured Alphaproteobacterium (59±14%) and two Gammaproteobacteria in UBA10353 (32±14%) and Thioglobaceae (9±6%).

**IDENTIFICATION OF DIFFERENTIALLY ABUNDANT MICROBES ASSOCIATED TO OCEAN ACIDIFICATION**

Differentially abundant microbial taxa are visualized in the heatmap in Figure S.3 (Gneiss linear regression model) based on correlation hierarchical clustering and metadata grouping by species and site. Changes across sites were evident in both sponges S. cunctatrix and C. reniformis. Multinomial regression models were computed in the Songbird pipeline for the identification of differentially abundant ASVs. In both sponge species the models displayed no overfitting when including ‘Site’ in the formula, and resulted in informative predictive accuracies (pseudo Q-squared values of 0.19 and 0.17 for C. reniformis and S. cunctatrix, respectively).

For the HMA sponge C. reniformis a total of 46 differentials were obtained, from which the top and bottom 20% were selected. This consortium fulfilled the defined thresholds for high associations to vent versus control sites (Figure 2.20). Positive centred log-ratios (red) represent taxa that were associated with the CO₂ vent. For log-ratio calculation, vent associated taxa were defined as numerator taxa, while control site associated taxa were considered in the denominator. Thus, ASVs which were negatively associated to the vent site showed a negative log-fold change (blue bars) and were more abundant in sponge individuals from the control site. The sample boxplot visualizes, that the natural log-ratios of vent samples were significantly higher than control samples. This
underlines that the selected numerator taxa are more abundant than the denominator taxa at the vent site (Welch’s Heteroscedastic F-Test ($p = 0.001$)).

Figure 2.20: *C. reniformis* - Selected log ratios of differentially abundant taxa ranked with respect to their association with the vent site. Data were obtained from the visualization tool ‘Qurro’. The divergent plot (A) shows the differentials, while the boxplot (B) visualizes their log fold change per Site. All 20/20 samples are shown containing top ranked features (20%) which showed a minimal log fold change of -1 or +1.

Figure 2.21: *S. cunctatrix* - Selected log ratios of differentially abundant taxa ranked with respect to their association with the vent site. Data were obtained from the visualization tool ‘Qurro’. The divergent plot (A) shows the differentials, while the boxplot (B) visualizes their log fold change per Site. A total of 17/20 samples are shown containing top ranked differentials (36%) with a log fold change of -1 or +1. The three missing samples belonged to the control site and could not be shown due to having an invalid (i.e. containing zero) log-ratio.

For the LMA sponge *S. cunctatrix* a total of 37 differentials were obtained, from which the top and bottom 36% were selected, with the predefined thresholds for high associations to vent versus
control sites (Figure 2.21). Similarly, the natural log-ratios of the selected features were significantly higher in the vent site compared to the control site (Welch’s Heteroscedastic F-Test (p < 0.001)).

**COMPOSITIONAL BETA DIVERSITY (DEICODE)**

Differences in compositional beta diversity was statistically significant between the sampling sites within sponge species (Permanova, p < 0.05). For each species, the DEICODE RPCA ordination yielded two clusters, one for the vent and one for the control site. Taxa that were the most significant drivers in *C. reniformis* and *S. cunctatrix* were represented as arrows in compositional biplots (Figure 2.22, Figure 2.23). Several features which explained the separation of sample groups in the DEICODE RPCA, were evaluated in taxa-to-taxa relationships with the tool ‘Quorro’. For the HMA sponge, *C. reniformis* differentials mostly accounting for beta diversity shifts across sites are presented in Figure 2.22. Using ‘Quorro’ I identified the following feature relationships with significantly different numerators/denominators: \( \frac{\text{Bdellovibrio}}{\text{SAR202}} \) (Figure S.4), \( \frac{\text{Endozoicomonas}}{\text{Burkholderiales}} \) (Figure S.5) and \( \frac{\text{Endozoicomonas}}{\text{Chloroflexus}} \) (Figure S.6). These taxa were selected based on results from DEICODE and Songbird differentials and were found to explain the best separation between the sample groups. ASVs belonging to the genus *Bdellovibrio* and *Endozoicomonas* were more associated with sponge samples from the vent, while SAR202, Burkholderiales and an uncultured Chloroflexus feature were associated to *C. reniformis* inhabiting the control site.

Differentially abundant taxa mostly accounting for beta diversity shifts in the LMA sponge *S. cunctatrix* across sites are shown in the DEICODE biplot in Figure 2.23. I identified the following significantly different numerators/denominators ratios of the following microbial orders: \( \frac{\text{UBA10353}}{\text{Zeaxanthinibacter}} \) (Figure S.7), \( \frac{\text{UBA10353}}{\text{Pseudomonadales}} \) (Figure S.8) and \( \frac{\text{SAR11}}{\text{Pseudomonadales}} \) (Figure S.9).

The UBA10353-to-Zeaxanthinibacter and the UBA10353-to-Pseudomonadales log-ratios were relatively high in control LMA sponge samples compared to the vent site. The genus UBA10353 was associated to the control site, while the genus Zeaxanthinibacter and the order Pseudomonadales were associated to the vent site.
Figure 2.22: Compositional DEICODE RPCA biplot of beta diversity from microbial communities associated to *C. reniformis* based on Aitchison distances. Circles represent individual samples from control and vent sites. Most relevant taxa driving differences in the ordination space are identified by vectors labelled with the respective taxonomy. Arrow directions indicate association of ASVs to vent or control community composition.

Figure 2.23: Compositional DEICODE RPCA biplot of beta diversity from microbial communities associated to *S. cunctatrix* based on Aitchison distances. Circles represent individual samples from control and vent sites. Most relevant taxa driving differences in the ordination space are identified by vectors labelled with the respective taxonomy. Arrow directions indicate association of ASVs to vent or control community composition.
DISCUSSION: MICROBIAL DYNAMICS OF HMA AND LMA SPONGE HOLOBIONTS IN RESPONSE TO OCEAN ACIDIFICATION

OCEAN ACIDIFICATION STUDIES IN A COMPLEX NATURAL ENVIRONMENT

This study aimed at estimating the influence of near-future OA scenarios on the diversity and composition of sponge associated microbiomes. Microbial phenotype responses of individuals of the HMA sponge *C. reniformis* and the LMA species *S. cunctatrix* living in a CO$_2$ vent with lowered pH, were compared versus specimens inhabiting in a control site with ‘normal’ pH. Natural CO$_2$ vents are considered as natural laboratories to assess the effects of OA (Cigliano et al., 2010; Hall-Spencer et al., 2008).

The two environments selected in this study maintained similar geophysical properties. Although revealing fluctuating nutrient concentrations, a similar order of magnitude was revealed without significant differences across both sites. Long-term logger data supported similar light conditions during the summer months, when sponge individuals and seawater samples were collected. During the autumn months, light intensity decreased in the control site with respect to the vent site. This could derive from its geographical localization off Ischia Island. The CO$_2$ vent site, situated in the South-East, receives more sun radiation during the winter months (Figure 2.26); whereas the control site situated in the North, experiences more shadow expositions.
Figure 2.24: Schematic representation of seasonal impact on the angle of sunlight over Ischia Island. The CO₂ vent site is represented as a red dot in the South-East, the control site in the North.

One notable difference between both study sites was the higher abundance of nano-planktonic communities at the CO₂ vent site, where *Synechococcus* spp. and picoeukaryotes revealed 2.5- and 3-fold higher cell concentrations, respectively. Former studies showed a fourfold increase in photosynthesis for *Synechococcus* when incubated in high CO₂ and temperature conditions (Fu et al., 2007). Mesocosm experiments further suggest, that CO₂ elevation significantly increases the abundance of photosynthetic picoeukaryotes (Shengnan Li et al., 2015). Since these nano-planktonic cells represent typical prey taxa of sponges (Cebrian et al., 2006; Morganti et al., 2016; Ribes et al., 1999), elevated planktonic abundances represent a higher food availability at the vent site. This likely represents a nutritional and energetical benefit for filter-feeders and might finally support holobiont fitness and sponge survival to withstand increased stress conditions under OA.

**Microbial alpha diversity**

Microbial diversity, on what regards species richness and evenness, was significantly higher in the HMA sponge *C. reniformis* than in its LMA counterpart, *S. cunctatrix*. This confirms the principle of the HMA-LMA dichotomy (Gloeckner et al., 2014), which classifies HMA sponges as having very dense and highly diverse microbial consortia (Reiswig, 1974; Vacelet and Donadey, 1977; Wilkinson, 1978b), versus LMA sponges as having generally lower microbial abundances (Erwin et al., 2011; Giles et al., 2013, 2013; Kamke et al., 2010; Moitinho-Silva et al., 2014a; Poppell et al., 2014; Schmitt et al., 2012; Weisz et al., 2007). The Sponge Microbiome Project dataset, including 575 sponge samples of 36 sponge species, also reported alpha diversity metrics generally greater in HMA than in LMA species (Moitinho-Silva et al., 2017b).
In response to OA, our results showed that microbial alpha-diversity increased in the LMA sponge and in seawater samples. Increases in gamma- and alphaproteobacterial diversity have been proposed to be correlated to coral reef sponges *lanthella basta* exposed to thermic stress (Luter et al., 2012). It was suggested that prior sponge mortality, increased diversity coincided with the breakdown in *I. basta*’s antimicrobial compound production, possibly allowing the proliferation of opportunistic bacteria. Accordingly, this could be a first indicator for a stress-induced dysbiosis of the symbiotic community associated to *S. cunctatrix* at the vent site. A mechanistical explanation could be, that since LMA sponges filter seawater more rapidly than HMA sponges (Ludeman et al., 2017; Perea-Blázquez et al., 2012; Weisz et al., 2008) and rely more heavily on horizontal symbiont transmission (Oliveira et al., 2020; Ribes et al., 2015), they take up more microbial cells from the environmental seawater. Together with the generally higher microbial abundance observed at the vent site, these phenomena entail the introduction of a larger variety of microorganisms in LMA sponges, including opportunist strains, which may contribute to increases in microbial abundances (Frank, 1996).

The HMA sponge *C. reniformis* showed an opposite trend and exhibited lower alpha diversities at the vent site compared to control pH conditions. Decreases in microbial diversity was also reported in 203 sponges sampled along a pollution gradient of environmental perturbation (Turon et al., 2019), suggesting that certain sponges under challenging environments might reflect impacts in their microbiomes. Ribes et al. (2016) reported no variations in overall microbial abundance, richness or diversity in aquarium experiments after exposing *Dysidea avara, Agelas oroides* and *C. reniformis* for a period of 66 days to near future pH conditions. Authors suggested that *C. reniformis* may have poor tolerance to OA, as no changes came along in the associated microbial communities, and no apparent growth was observed under acidified conditions. In contrast, our results do unveil certain variability in the microbial composition in *C. reniformis* inhabiting control versus vent sites. These contrasting outcomes likely rely on the different experimental settings: short-term aquarium experiments versus long-term adapted holobionts living at CO$_2$ vents.

Responses of free-living microbial communities in seawater towards OA is poorly understood. Mesocosm experiments on picoplankton communities under ‘high-CO$_2$’ conditions (∼750 ppm), simulating future OA predictions for the year 2100 over time periods of 18 and 20 days found little evidence for bacterial abundance or compositional changes (Newbold et al., 2012). Our data instead found that three alpha diversity metrics (observed features, Shannon, Faith’s PD) increased at the vent site in comparison with the control site. Increased bacterial production was reported at very high pCO$_2$ levels ranging between 2000 and 10 000 µatm in aquarium experiments (Yamada et al., 2008). Other mesocosm studies, mimicking pCO$_2$ levels of predicted future scenarios, revealed that the total abundance of bacteria varied due to phytoplankton blooms been triggered in response to OA, but they found little evidence for direct effects of pCO$_2$ on bacterial abundances

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Indeed, it is hypothesized that autotrophs, which have carbon-concentrating mechanisms (CCM) may profit from increased CO₂ availability by reduction for CCM activity and reduced allocation of energy or nutrients required for carbon acquisition, affecting photosynthesis and growth rates (Beardall and Giordano, 2002; Burkhardt et al., 2001). In our study, photosynthetic phytoplankton cells, such as the cyanobacterial Synechococcus were more abundant at the vent site. Increased growth rates and photosynthesis under acidified conditions were shown for the cyanobacterium Trichodesmium spp. (Barcelos e Ramos et al., 2007), as well as for the cyanobacterial Synechococcus sp. and Prochlorococcus sp. (Fu et al., 2007).

Generally, care has to be taken when transferring knowledge obtained from laboratory mesocosm experiments to natural environments. Mesocosm experiments induce artificially lowered pH conditions for short-term or mid-term periods, which do not allow realistic estimations of microbial shifts to OA. Volcanic CO₂ vent systems are actually ideal experimental settings to assess the long-term effects of high pCO₂ and reduced pH on microbial processes (O’Brien et al., 2016).

**MICROBIOME COMPOSITION**

The microbiome composition was significantly different between both sponge species and with respect to the seawater. This outcome was expected, since sponges despite sharing similar microbial phyla, do maintain a high degree of host specificity (Moitinho-Silva et al., 2017a; Schmitt et al., 2012). In 2002, Hentschel et al. described that sponges share highly similar bacterial phyla by showing that sponge-derived 16S rRNA gene sequences clustered together in a large array of hosts. But on lower taxonomical levels, different sponges are characterized by species-specific communities and share few bacterial taxa (Hentschel et al., 2002).

The microbiome of *C. reniformis* and *S. cunctatrix*, comprised HMA and LMA indicator taxa, in agreement with a study which characterized the microbiomes of 19 HMA and 17 LMA sponges (total 575 specimens) in the frame of the Sponge Microbiome Project (Moitinho-Silva et al., 2017a). The bacterial phyla Chloroflexi, Poribacteria and Acidobacteria were more abundant in HMA sponges, whereas LMA sponges showed higher abundance in Proteobacteria and Bacteroidetes. At the class level, the general trend was higher abundance of SAR202, Anaerolineae and PAUC34 in HMA species, and larger preponderance of Alphaproteobacteria (up to 3 times higher) in LMA sponges. Our findings revealed class Acidimicrobia, in general more abundant in HMA sponges, as being more abundant in *S. cunctatrix*. Cyanobacteria was found to be more preeminent in the LMA species and in the seawater, and absent in *C. reniformis*. 
COMPOSITIONAL SHIFTS IN RESPONSE TO OA

OA induced compositional changes in both sponge species. When comparing individuals from vent and control sites, separated clusters of microbial features were obtained applying various beta diversity metrics. Relative abundances of microbial taxa shifted at broad taxonomical levels – from phylum down to genus level. Changes in the microbial composition seem to be species-specific. Earlier explorations examined the response of two different coral reef sponges *Coelocarteria singaporesis* and *Stylissa cf. flabelliformis* between a control pH reef site and an adjacent CO$_2$ vent in Papua New Guinea (Kandler et al., 2018; Morrow et al., 2015). While the microbial composition of *C. singaporesis* differed significantly between sites, the microbiome of *S. cf. flabelliformis* was rather conserved. Similarly, OA experiments in aquarium conditions showed no significant changes in microbial community composition of the iconic barrel sponge, *Xestospongia muta* (Lesser et al., 2016).

Some taxa shift trends observed in *C. reniformis* and *S. cunctatrix* microbiomes were analogous to those reported in sponges submitted to transplant experiments performed at Papua New Guinea vents (Kandler et al., 2018). Phyla Chloroflexi and Acidobacteria decreased and Proteobacteria increased in relative abundance in *C. reniformis* living under OA conditions, similar as in the HMA species *C. singaporesis* after transplantation to the vent area (Kandler et al., 2018; De Menezes et al., 2022; Waterworth et al., 2021). On top of this, Proteobacteria decreased and Bacteroidota increased in abundance in *S. cunctatrix* from the vent site, as did in the LMA sponge *Stylissa cf. flabelliformis*, when transplanted to the vent (Glasl et al., 2018; Kandler et al., 2018).

Microbial populations in the water column reported shifts, with increases in Gammaproteobacteria and Flavobacteriacea in seawater samples coming from the vent site, and decreases in Alphaproteobacteria. Family Rhodobacteraceae, instead was stable across both sites. These outcomes are similar to prior studies with free-living marine microorganismal communities. A highly-replicated laboratory experiment with the natural bacterial community from Helgoland Roads (North Sea), showed that small changes in pH had direct effects on the bacterial community composition. They identified Gammaproteobacteria, Flavobacteriacea, Rhodobacteraceae and Campylobacteracea as phylogenetic groups responding most notably to differences in pH (Krause et al., 2012).

The beta dispersion, as the variability in microbial community composition within groups was smaller than that between groups in *C. reniformis*. This suggests that across sites, the microbiome composition was different, but showed stability among *C. reniformis* individuals inhabiting the same site. Instead, *S. cunctatrix* showed significant differences in beta dispersions per site. This increased inter-individual (intragroup) variation in microbial compositions supports a seeming status of destabilization and dysbiosis in *S. cunctatrix* living under acidified conditions. Stress-related increases in sponge microbiome beta dispersion have been observed in manipulative experiments.
(Lesser et al., 2016; Pineda et al., 2017), as well as under natural perturbations (Erwin et al., 2012; Turon et al., 2019). These observations are consistent with the recently proposed ‘Anna Karenina principle’, which describes that intraspecific variability is higher in dysbiotic than in healthy individuals, paralleling Leo Tolstoy’s dictum ‘all happy families look alike; each unhappy family is unhappy in its own way’ (Zaneveld et al., 2017).

**CORE MICROBIOME TRENDS**

Microbial communities can be divided into a core microbiome and a variable microbiome. A core microbiome is comprised of members which are highly prevalent among a defined habitat (Hamady and Knight, 2009; Turnbaugh et al., 2007). In our analyses, this means the consistent presence of microbial features among all host individuals of the same sponge species, or sharing a particular grouping condition? (Pita et al., 2018; Shade and Handelsman, 2012). Identifying core microbes is essential to understand the response of a microbial community under environmental perturbations, such as OA, since the core microbiome seems to play key functions for the overall microbial ecosystem (Astudillo-García et al., 2017; Cárdenas et al., 2014; Shade and Handelsman, 2012). The identification of a core can help to classify a ‘healthy’ community which might support overall tolerance of the sponge holobiont to OA and give insights into dysbiosis vs. compositional acclimatization (Pita et al., 2018).

**SHARED MICROBIOMES AND CORE SIZE**

Comparing the same sponge species inhabiting the vent and control site revealed that a large proportion of ASVs were unique across sites, suggesting symbiont switching (see Venn diagram in Figure 2.17). LMA sponges shared more than 20% of their microbial taxa with the surrounding seawater, while the HMA sponges shared no core with the seawater. Both sponges shared some sponge-typical microbes belonging to Alphaproteobacteria, Bacteroidia and Gammaproteobacteria. In a prior study from Erwin and co-workers (Erwin et al., 2015), a great amount of ASVs were shared between the seawater, *C. reniformis* and *S. cunctatrix*. Our results suggest a stronger host-specificity in *C. reniformis*. OA decreased the number of shared ASVs between the LMA and HMA sponges by 90% (from 35 down to 4), while a big number of shared features were maintained across LMA sponges and the seawater. This may indicate a further sign for a stress-induced dysbiosis in *S. cunctatrix* due to a disruption of the symbiotic community by shifting from sponge-enriched microbes (closely related to other sponge symbionts, here the HMA sponge) towards opportunists, i.e. microbes closely related to free-living organisms in the seawater (Pita et al., 2018; Simister et al., 2012b). Since the composition of core microbiomes changed notably in OA conditions, it is not surprising that core sizes decreased when including both environments in core microbiome analyses. This was
especially true for the core size of *C. reniformis*, where few overlapping core taxa were found across both sites. This may suggest a higher microbial restructuring of the core microbiome at the vent site, which might support acclimatization and even adaptation to OA (Pita et al., 2018; Ribes et al., 2016; Webster and Reusch, 2017). In contrast, the LMA sponge showed only a slight decrease in the core size, when comparing the core microbiome comprising both sites against only the control group. When taking a look at the core diversity, this measure notably increased in *S. cunctatrix* from the vent site. These events, along with the enhanced richness observed in *S. cunctatrix* living at the vent site, are likely correlated with the increased microbial diversity recorded in the vent ambient seawater, and the wide range of taxa shared with the sponge. The higher water pumping rates and filtering capacities of *S. cunctatrix* could probably favour the incorporation of new microbes from the surrounding environment, in this case at the vent site, with respect to the HMA counterpart.

**CORE COMPOSITIONAL SHIFTS, CORE FUNCTIONS AND HOLOBIONT RESILIENCE**

Microbially mediated acclimatization in sponge hosts can be performed via different strategies. On the one hand endosymbiotic microorganisms can change their genetic information much faster than higher host organisms, since they have much shorter generation times and thus a faster response to environmental changes (Reshef et al., 2006; Ribes et al., 2016). On the other hand, a sponge host can simply acquire new microbes from the environment and re-shape the composition of its microbiome. Taking in consideration the functional roles core microbes can perform within their sponge holobiont, I will proceed to discuss key core taxa which were stable across both sites for each species, and then identify core ASVs which were either vent or control site associated. A literature review was conducted to assign putative core functions within the target sponge hosts. This approach aimed at estimating if compositional changes of core microbiomes could be ascribed to microbial-related acclimatization processes.

By definition, core functions are microbially-mediated metabolic features which are often performed by analogous pathways, and which are particularly relevant for symbiosis and holobiont success (Fan et al., 2012; Hentschel et al., 2012; Horn et al., 2016; Pita et al., 2018; Ribes et al., 2012; Thomas et al., 2010). These microbial functions are found among multiple, geographically distant sponge species, and are characterized by functional convergence (Thomas et al., 2016). Often core functions involve housekeeping genes, which are necessary for microbial life. But they also contribute to metabolic and defensive strategies that allow the sponge microbiota to colonize, interact and adapt to a host environment, and at the same time, support sponge host nutrition, biogeochemical cycling and chemical defense strategies (Pita et al., 2018).

Most core functions that were introduced at the vent site in both, HMA and LMA sponges were related to the utilization of environmental and host derived nutrients. Introduced taxa showed certain functional convergence with core microbes that were lost at the vent site (metabolic
redundance), supporting the maintenance of ecosystem functioning of the sponge holobiont (Erwin et al., 2012; Ribes et al., 2012; Thomas et al., 2010). Especially in the HMA sponge, the more diverse core microbiome and its compositional changes had impact on a wide range of associated core functions, including pathways for carbon-, nitrogen-, and sulfur-metabolism, vitamin synthesis and secondary metabolite production, which may play a role in defense strategies and microbe-microbe interactions. A summary of possible core functions of introduced or stable core taxa between CO$_2$ vent and control sites is provided in supplementary tables S.1-S.4. These functions are speculative and need to be tested in futures studies.

**SPONGE-ASSOCIATED MICROBIOME DYNAMICS UNDER OCEAN ACIDIFICATION**

Core microbiome analyses revealed stable core features among both sites, as well as newly introduced or lost features. Several compositional data analyses tools were used to identify differentially abundant ASVs and reveal their association to CO$_2$ vent sites or control pH conditions. Interestingly, many core symbionts were found among differentially abundant features with higher abundance in either control pH or acidified conditions. This underlines the key role and highly beneficial association to core symbionts and their performed core functions, which have “likely coevolved to maintain host functionality and fitness over ecological and evolutionary time scales” (Björk et al., 2018). An intraspecific self-regulation and maintenance of associated core symbionts seems to be much more important for microbiome dynamics than environmental pressures. But, when core symbionts are lost in response to environmental perturbations, the holobiont system may reach a new healthy state by compensation with other taxa, that are able to maintain core functions and promote acclimatization to the new conditions (Pita et al., 2018). Table 2.9 summarizes all highly ranked features with assigned putative functions according to current literature review, where microbial functions were mainly provided from culturing experiments or single-cell genomic analyses. In the following sections, I will discuss in detail key functions of identified core microbes, which were found to be conserved across both sites and mention if there were higher abundance shifts to a specific site. Then, differentially abundant features with association to control pH or vent sites will be summarized with the corresponding putative metabolic functions. Interpretations are discussed separately per species, since it is widely accepted that the microbiome and microbial responses are species-specific (Engelberts et al., 2020; Hentschel et al., 2012; Moitinho-Silva et al., 2017a; Reveillaud et al., 2014).
C. reniformis

Stable Core Microbiome in C. reniformis Across Acidified and Control Sites

Stable core microbiota of the HMA sponge C. reniformis, revealed five key taxa with a broad range of metabolic potential. The Gammaproteobacteria genus Endozoicomonas was the most dominant core microbial group across both sites, with increasing abundance at the CO₂ vent. Endozoicomonas are classified as extremely diverse and flexible endosymbionts (Neave et al., 2016) with broad metabolic potentials, which likely support host health and resilience under OA via different mechanisms. Besides participating in basic host metabolism, including host-associated protein and carbohydrate transport and cycling, as well as DOM consumption (Campana et al., 2021), this symbiont directly plays a role in amino acid production (Neave et al., 2016, 2017). Endozoicomonas can also effectively metabolize DMSP into DMS, while using DMSP as a carbon source for growth and survival. Interestingly, DMSP and DMS play a role in oxidative and osmotic stress protection (Lesser, 2006; Tandon et al., 2020). During the catabolism of DMSP antimicrobial compounds are produced (tropodithietic acid), which were shown to inhibit the growth of pathogens in corals (Peixoto et al., 2017; Raina et al., 2016). The extremely diverse metabolic potential of Endozoicomonas is explained by its large genome size (Neave et al., 2016), with a high degree of functional adaptation and genomic plasticity supported by a large proportion of transposable elements (Neave et al., 2016, 2017). Larger genome sizes are generally attributed to higher levels of horizontal gene transfer (HGT) (Horn et al., 2016; Pita et al., 2018) and may play a role in the evolutionary adaptation of the sponge microbiota to a symbiotic lifestyle (Fan et al., 2012; Gao et al., 2014; Thomas et al., 2010). In turn, HGT is likely to support the acquisition of novel traits and functions to affect holobiont phenotypes leading towards acclimatization (Pita et al., 2018).

Two further stable core microbes belonged to the phylum Chloroflexi, and were SBR1031 (A4b) and SAR202. SAR202 showed higher abundance under control pH conditions. Both core representatives have genes involved in nitrogen metabolism and thus support biogeochemical cycling and host nutrition (Kamke et al., 2013; Slaby et al., 2017). SBR1031 are considered to be anaerobic nitrifying symbionts (Li et al., 2020; Yamada et al., 2006) and able to degrade polycyclic aromatic hydrocarbons (PAHs), which are prevalent organic pollutants in coastal ecosystems (Wu et al., 2022). The symbiont SAR202 is also able to degrade complex carbohydrates (Thrash et al., 2017), including PAHs (Colatriano et al., 2018; Wang et al., 2020), reduce nitrate (Thrash et al., 2017) and play a major role in sulphur turnover via sulphite oxidation (Mehrshad et al., 2018). Interestingly, this symbiont plays a central role in the Sponge Loop (De Goeij et al., 2013) via DOM consumption (Campana et al., 2021) and the degradation of labile and recalcitrant DOM (Bayer et al., 2018; Landry et al., 2017). This enhances DOM recycling and nutrient supply for the sponge host. Additionally, SAR202 contain CRISPR-Cas systems, eukaryote-like repeat proteins, and secondary
metabolite gene clusters (Bayer et al., 2018), which might support an intrinsic defense against viruses or phages (Horn et al., 2016; Pita et al., 2018; Slaby et al., 2017; Thomas et al., 2010). Genus *Bdellovibrio* was also found among the stable core microbiome across both sites with higher differential abundance under acidified conditions. This symbiont can be classified as an 'amphibiotic' biocontrol agent, having probiotic and 'living antibiotic' properties. As an obligate predatory bacteria they prey non-specifically on numerous bacterial gram-negative pathogens (Cavallo et al., 2021; Harini et al., 2013; Jurkevitch, 2012; Koval et al., 2013; Markelova, 2010; Starr and Baigent, 1966). This feeding behaviour likely regulates pathogen levels within the sponge holobiont, supporting overall holobiont health. Simultaneously, *Bdellovibrio* are non-pathogenic to higher (host) organisms (Dwidar et al., 2012).

Another stable core feature appertained to Gemmatimonadota, which is common in terrestrial environments (DeBruyn et al., 2011). This taxon was also found with relatively high abundance in the HMA sponge *Haliclona cnidata* (Schellenberg et al., 2020). These microbes are able to acquire genes via HGT (Zeng et al., 2014), supporting genetic plasticity and a putative adaptation to changing environmental parameters (Pita et al., 2018). Conclusively, stable symbiotic partnerships with the above core taxa across both, acidified and control sites, suggest that these taxa perform key metabolic functions for overall ecosystem functioning. Due to their versatile and beneficial core functions they likely support resilience of *C. reniformis* to OA (Erwin et al., 2015; Webster, 2007).

**CONTROL PH ASSOCIATED SYMBIONTS IN C. RENIFORMIS**

The core symbiont *Poribacteria* disappeared from the vent core in *C. reniformis* and showed general association to control pH conditions. It is possibly an ancient symbiont and is nearly exclusively found in marine sponges (Siegl et al., 2011). Besides being involved in mediating sponge-microbe interactions and autotrophic CO₂ fixation (Siegl et al., 2011), it performs Vitamin B1 synthesis (Engelberts et al., 2020) and DOM consumption (Campana et al., 2021). Other typical sponge symbionts PAUC34 and Myxococcota were core microbes associated to control pH conditions and were similarly lost at the vent site. These symbionts possibly contribute to the degradation of complex carbohydrates (Astudillo-García et al., 2018; Thrash et al., 2017) and secondary metabolite production (Gemperlein et al., 2018; Herrmann et al., 2017) such as antibiotics (Schäberle et al., 2014) in *C. reniformis* at control sites, respectively.

**VENT ASSOCIATED SYMBIONTS IN C. RENIFORMIS**

The introduction of new core microbes at the vent site (i.e., features belonging to Dadabacteriales, Nitrospira and Constrictibacter) were likely compensating for functional losses. For instance the lost functions attributed to *Poribacteria*, could be balanced by photoheterotrophic Dadabacteriales, which report genes for Vitamin B1 synthesis (Engelberts et al., 2020) and the ability to degrade DOM (Graham and Tully, 2021), supporting host nutrition and health. Additionally, this genus
contains genes for steroid precursor biosynthesis which could provide the host with diverse secondary metabolites (Engelberts et al., 2020; Hoshino and Gaucher, 2021), supporting microbiome interactions and chemical defense strategies (Fan et al., 2012; Fiore et al., 2015; Thomas et al., 2010). Dadabacteriales were also found to have increased abundances among bleached corals (Kusdianto et al., 2021). Additionally, this genus experienced increases in abundance in the sponge *Carteriospongia foliascens* after stress treatments under elevated temperature and pCO$_2$ (Luter et al., 2020).

*Nitrospira* is involved in nitrogen cycling via the oxidation of ammonia (Mohamed *et al.*, 2010; Simister *et al.*, 2012b) and nitrite (Burgsdorf et al., 2022; Hoffmann et al., 2009; Simister *et al.*, 2012b). This genus additionally participates in autotrophic carbon-fixation (Burgsdorf et al., 2022) and DOM consumption (Campana *et al.*, 2021), supporting energy supply with nutrients (Kamke *et al.*, 2013; Slaby *et al.*, 2017; Webster and Thomas, 2016).

The genus *Constrictibacter* metabolizes organic compounds such as ribose, pyruvate and succinate (Yamada *et al.*, 2011) and may contribute to overall carbon metabolism of the holobiont ecosystem. This genus was also found to be among the core microbiome of the Caribbean sponge *Ircinia*, as putatively vertically transmitted symbionts (Kelly *et al.*, 2021).

Another highly vent associated feature was *Candidatus Kaiserbacterium*. This feature belongs to the class Parcubacteria and the phylum Patescibacteria. The reduced genome of *Candidatus Kaiserbacterium* comes along with severely reduced metabolic capabilities, which reflects a signature for a symbiotic lifestyle (Rahlf et al., 2020). While having housekeeping genes, it lacks genes for the biosynthesis of cofactors, amino acids, nucleotides, and fatty acids (Nelson and Stegen, 2015). It needs to tap into the microbial community for resources like lipids and DNA (Bayer *et al.*, 2020). Besides being classified as symbionts, Parcubacteria also show signatures for free-living, streamlined organisms with parasitic and commensal lifestyles (Nelson and Stegen, 2015). As ecosymbionts or parasites, some strains within Parcubacteria attach to external surfaces of other microbial cells to facilitate access to nutrients and energy sources (Nelson and Stegen, 2015). However, the sponge host might benefit from *C. Kaiserbacterium* through its participation in nitrogen metabolism by reducing nitrate (Danczak *et al.*, 2017), as well as lactate and malate fermentation (Vigneron *et al.*, 2020). The presence of *C. Kaiserbacterium* in acidified conditions was further reported in biofilms at a hydrothermal spring cave (Anda *et al.*, 2020).
**S. cunctatrix**

**Stable core microbiome in S. cunctatrix across acidified and control sites**

The stable core microbiome of *S. cunctatrix* across sites was comprised by three features belonging to classes Alpha- and Gammaproteobacteria. Core microbes belonging to the class Gammaproteobacteria seemed to comprise important symbionts for chemosynthetic symbioses (Hestetun et al., 2016; Rubin-Blum et al., 2019; Zhou et al., 2019). The alphaproteobacterial core ASV remained unclassified, and as a consequence, associated functions unrevealed. The most dominant Gammaproteobacterium, belonging to the genus UBA10353, was present among the core microbiome at both sites, but with higher abundance in control conditions. This symbiont is likely involved in the carbon and sulfur metabolism of the sponge host via chemoautotrophic carbon fixation and thiosulfate oxidation (Burgsdorf et al., 2022). This genus was also found to fix dissolved inorganic carbon in the dark ocean and contains genes for taurine utilization (Baltar et al., 2022). It also harbours biosynthetic gene clusters for the production of a pederin-type secondary metabolite, ‘mycalamide’, which has antiviral, apoptotic (Hood et al., 2001) and protein synthesis inhibiting effects (Dyshlovoy et al., 2012; Rust et al., 2020). This core microbe was also found to be dominant in the sponge *Spinularia sp.* inhabiting peripheral areas of deep-sea hydrothermal vents (Georgieva et al., 2020).

The second most abundant gammaproteobacterial core symbiont, belonging to the family Thioglobaceae, was also a chemosynthetic, sulfur-oxidizing symbiont (Ansorge et al., 2020) and was previously found in the sponges *Cladorhiza* and *Spinularia sp.* inhabiting peripheral areas of deep-sea hydrothermal vents (Georgieva et al., 2020), as well as in in healthy octocorals (Keller-Costa et al., 2022). Thioglobaceae associated with sponges from deep-sea chemosynthetic environments, are known to perform sulfide and/or methane oxidation (Rubin-Blum et al., 2019; Zhou et al., 2019). Consequently, gammaproteobacterial core microbes associated to *S. cunctatrix* are likely to be main sulfide-oxidizing symbionts, analogously to those found in vent and seep fauna (Dubilier et al., 2008).

**Control pH associated symbionts in S. cunctatrix**

Features belonging to the family Cryomorphaceae were revealed as more associated to the control site for *S. cunctatrix*. This bacterial family is usually enriched in the marine surface layer (Bowman, 2020; Zäncker et al., 2018) and occurs in a range of marine habitats, including sediment and sea ice (Bowman, 2020; Parte et al., 2011; Zäncker et al., 2018). Some genera of this family may exhibit photoheterotrophy (Bowman, 2020; Gómez-Consarnau et al., 2019) and grow at low temperatures, e.g. Antarctica, but also in tropical regions (Parte et al., 2011). Cryomorphaceae cannot utilize carbohydrates and require complex organic compounds for growth (Bowman et al., 2003; Lau et
Mutualistic relationships with Antarctic phytoplankton blooms were reported (Delmont et al., 2015). Contrasting the observed trends in *C. reniformis*, where Dadabacteriales were highly associated to acidified conditions, this symbiont was more closely associated to *S. cunctatrix* at the control site. As earlier discussed, this symbiont is likely supporting holobiont functions via DOM degradation (Graham and Tully, 2021), steroid precursor biosynthesis (Hoshino and Gaucher, 2021) and Vitamin B1 synthesis (Engelberts et al., 2020).

The alphaproteobacterial clade SAR11 was associated to *S. cunctatrix* samples from the control site, although in reported short-term OA experiments (using hydrochloric acid and sodium bicarbonate for acidification), SAR11 showed a high physiological resilience to OA (Hartmann et al., 2016). This heterotrophic clade typically dominates bacterioplankton (Hartmann et al., 2016; Simister et al., 2012b), representing a nutrient-rich food source for the smallest eukaryotes (Hartmann et al., 2013; Sherr and Sherr, 2002), while demineralizing phytoplankton-derived organic matter (Boyd et al., 1999; Martin et al., 1987). Since the relative abundance of SAR11 dropped sharply in seawater samples at the vent site, the above pattern may be reflected in the microbiome of *S. cunctatrix*, as a result of filter-feeding (horizontal) acquisition.

**Vent Associated microbes in *S. cunctatrix***

Several core microbes were introduced in *S. cunctatrix* living at vent conditions. Two ASVs belonged to the order Flavobacteriales, including genus *Zeaxanthinibacter* and an uncultured microbe belonging to the family Cyclobacteriaceae. Flavobacteriales were particularly predominant in seawater samples at the vent site. Increases of Flavobacteriales were also reported in biofilms from the Great Barrier Reef at increased CO$_2$ concentrations (Witt et al., 2011). Also mesocosm experiments confirmed increased relative abundance of Flavobacteriaceae in bacterioplankton under synergistic effect of acidification and nutrient enrichment (Baltar et al., 2015). As symbionts *Zeaxanthinibacter* are likely involved in sulfur metabolism (Declercq et al., 2013), carotenoid pigment production (Asker et al., 2007) and the production of secondary metabolites with antifungal activity (Sang and Kim, 2012). Certain Flavobacteriales species are responsible for a severe fish disease causing skin lesions, fin erosion and gill necrosis (Declercq et al., 2013) via the degradation of complex acidic polysaccharides of connective tissues (Declercq et al., 2013). It can be speculated that the increased presence of Flavobacteriales as core microbiota might correlate with the impaired morphology and signs of necrosis of *S. cunctatrix* at the vent site. The core feature belonging to Cyclobacteriaceae could be involved in DOM consumption (Campana et al., 2021), providing the holobiont with nutrients (Kamke et al., 2013; Slaby et al., 2017; Webster and Thomas, 2016).
A cyanobacterium was introduced as core microbe of S. cunctatrix at the vent site. Cyanobacteria are reported to participate in nitrogen and carbon fixation (diazotrophs and photosynthesis), providing the sponge holobiont with nutrients (Bednarz et al., 2017; Durall and Lindblad, 2015; Klawonn et al., 2016). Under OA primary production of cyanobacteria is likely to increase, as described in *Trichodesmium* cell lines adapted to high-CO$_2$ (Walworth et al., 2016). These mechanisms may provide *S. cunctatrix* with a higher amount of nutrients to maintain physiological performance at the CO$_2$ vent.

AqS1, a sulfur- and ammonia-oxidizing Gammaproteobacterium was not part of the core microbiome, but showed higher abundances within the *S. cunctatrix* microbiome from the vent site. These microorganisms might mediate beneficial metabolic functions and support overall holobiont acclimatization, by providing energy supply and support sponge reproduction (Lavy et al., 2018; Taylor and Kurtz Jr., 2020). As a chemoautotrophic symbiont, AqS1 is able to oxidize sulfide to sulfate and supply the host with energy, as shown in the sponge *Amphimedon queenslandica* (Gauthier et al., 2016). Sulfate further seems to represent a significant nutritional resource for the sponge microbiome, while sulfide is required to compose amino acids, vitamins, secondary metabolites and sulfo-lipidic compounds (Lavy et al., 2018). Unlike their sponge host, it was shown that AqS1 is capable of synthesizing arginine from citrulline (Gauthier et al., 2016) and supply arginine back to their host (Song et al., 2021). Arginine is then used for NO production, which is critical for larval settlement. Interestingly, AqS1 was found to be especially abundant in the inner cell mass of *Amphimedon queenslandica* larvae (Song et al., 2021), and was suggested to be along with AqS2 and AqS3, likely vertically transmitted (Fieth et al., 2016). Due to playing a critical role in sponge development, larval settlement and metamorphosis, AqS1 could likely be proposed to play a role in supporting the life cycle and survival of *S. cunctatrix* under vent conditions.

**COMMON PATTERNS IDENTIFIED AMONG BOTH SPONGE SPECIES**

The symbiont SAR86 was more abundant in control pH conditions in both sponge species, *C. reniformis* and *S. cunctatrix*. Also, relative abundances were higher in seawater samples. This Gammaproteobacterium belongs to the order Pseudomonadales and is often found in surface ocean bacterioplankton (Dupont et al., 2012; Rappé et al., 2000; Simister et al., 2012b), and is particularly abundant in algal blooms (González et al., 2000). The SAR86 clade is a very diverse clade (Suzuki et al., 2001; Treusch et al., 2009) and seems to flourish only in oligotrophic *Prochlorococcus* dominated waters (Fuchs et al., 2005). Their genomes lack several pathways for amino-acid and vitamin synthesis as well as sulfate reduction (Dupont et al., 2012), favouring a symbiotic lifestyle. Since this symbiont was not cultivated until today, there is scarce knowledge about its potential functions, but it is suggested that functional capabilities vary greatly across locations (Dupont et al., 2012; Hoarfrost et al., 2020; Rusch et al., 2013; Swan et al., 2013).
One feature belonging to the family Thermoanabaculaceae was found to be associated to the vent site in both sponge species. Unfortunately, little is known about the metabolic potential of this bacterial family. They are classified as anaerobic chemoheterotrophs mainly found in freshwater hot springs (Dedysh et al., 2020; Dedysh and Yilmaz, 2018), but also in arctic sediments (Walker et al., 2021). *Thermoanaerobaculum aquaticum* is the only described member of the family Thermoanabaculaceae (Dedysh et al., 2020), and has reported optimal growth at pH 6.0 – 8.0, with an optimum between pH 6.5 and 7.0 (Losey et al., 2013). Also *Thermotomaculum hydrothermale*, a heterotrophic thermophile within the phylum Acidobacteria was found in deep-sea hydrothermal vents, displaying growth at pH 5.5 - 8.5, with an optimum recorded at pH 6.6 (Izumi et al., 2012). The optimal growth rates of Acidobacteria in rather acidic environments might have favoured their reproduction within the holobiont at the vent site, but further investigation is needed to estimate their role within their sponge host under OA.
Table 2.4: Summary of identified differentially abundant microbes and their putative functions. Listed are microbial taxa identified with bioinformatic tools: RA: Relative Abundance; SB: Songbird; DC: Deicode Differential Beta Diversity and DS2: DeSeq2. Arrows represent increased (↑) or decreased (↓) correlation with the vent site. If nothing is specified for DS2 same results were obtained as suggested with other tools; contradictory result is specified with a different arrow representing either up- or downregulation. Presence or absence among the core microbiomes are indicated, with arrows indicating increased or decreased relative abundances across the core. A literature review of putative functions is provided.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Method</th>
<th>Core</th>
<th>Vent</th>
<th>Putative Functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bdellovibrionota</em></td>
<td>RA, SB,</td>
<td>CM,</td>
<td>↑</td>
<td>Symbiosis 'Amphibiotic' biocontrol agent → probiotic and 'living antibiotic': prey on numerous gram-negative pathogens.</td>
<td>(Cavallo et al., 2021; Harini et al., 2013; Jurkevitch, 2012; Koval et al., 2013; Markelova, 2010; Starr and Baigent, 1966)</td>
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<tr>
<td><em>Bdellovibrio</em></td>
<td>DS2</td>
<td>CV</td>
<td></td>
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<tr>
<td><em>Patescibacteria</em></td>
<td>RA, SB,</td>
<td></td>
<td>↑</td>
<td>Metabolism and Nutrition Lactate dehydrogenase and malate fermentation</td>
<td>(Vigneron et al., 2020)</td>
</tr>
<tr>
<td><em>Parcubacteria</em></td>
<td>DC, DS2</td>
<td></td>
<td>↑</td>
<td>Nitrile reduction and encode putative proton-translocating hydrogenase and an ATP synthetase</td>
<td>(Danczak et al., 2017)</td>
</tr>
<tr>
<td><em>C. Kaiserbacteria</em></td>
<td></td>
<td></td>
<td></td>
<td>Genome 'Taker' microbe: reduced genome and metabolic capabilities (symbiotic, parasitic, and commensal lifestyle)</td>
<td>(Bayer et al., 2020; Nelson and Stegen, 2015; Rahlf et al., 2020)</td>
</tr>
<tr>
<td><em>Dadabacteria</em></td>
<td>RA, SB,</td>
<td>CM,</td>
<td>↑</td>
<td>Metabolism and Nutrition Degradation of microbial DOM (peptidoglycan and phospholipids)</td>
<td>(Graham and Tully, 2021)</td>
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<tr>
<td><em>Dadabacteriales</em></td>
<td>DS2</td>
<td>CV</td>
<td></td>
<td>Symbiosis Increased in bleached corals</td>
<td>(Kusiakato et al., 2021)</td>
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<td></td>
<td></td>
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<td></td>
<td>Higher relative abundance in the elevated temperature and pCO2 treatments in the sponge <em>C. foliacea</em></td>
<td>(Luter et al., 2020)</td>
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<td></td>
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<td></td>
<td></td>
<td>Genome Small genome size</td>
<td>(Graham and Tully, 2021)</td>
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<td>Secondary Degradation of microbial DOM (peptidoglycan and phospholipids)</td>
<td>(Hoshino and Gaucher, 2021)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Metabolites Steroid precursor biosynthesis via mevalonate pathway, similar to eukaryotic pathways</td>
<td>(Engelberts et al., 2020)</td>
</tr>
<tr>
<td><em>Proteobacteria</em></td>
<td>RA, SB,</td>
<td>CM,</td>
<td>↑</td>
<td>Metabolism and Nutrition Protein and carbohydrate transport and cycling; Production of amino acids</td>
<td>(Neave et al., 2014, 2016, 2017)</td>
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<tr>
<td><em>Gammaproteobact.</em></td>
<td>DC, DS2</td>
<td>CV</td>
<td></td>
<td>DOM consumption, Nitrate assimilation</td>
<td>(Campana et al., 2021)</td>
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<tr>
<td><em>Pseudomonadales</em></td>
<td></td>
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<td></td>
<td>Symbiosis Increased in bleached corals</td>
<td>(Kusiakato et al., 2021)</td>
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<td><em>Endoaizomonas</em></td>
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<td>Higher relative abundance in the elevated temperature and pCO2 treatments in the sponge <em>C. foliacea</em></td>
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<td>Secondary Degradation of microbial DOM (peptidoglycan and phospholipids)</td>
<td>(Hoshino and Gaucher, 2021)</td>
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<td>Metabolites Steroid precursor biosynthesis via mevalonate pathway, similar to eukaryotic pathways</td>
<td>(Engelberts et al., 2020)</td>
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<td>Symbiosis Increased in bleached corals</td>
<td>(Kusiakato et al., 2021)</td>
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<td>Higher relative abundance in the elevated temperature and pCO2 treatments in the sponge <em>C. foliacea</em></td>
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<td>Metabolites Steroid precursor biosynthesis via mevalonate pathway, similar to eukaryotic pathways</td>
<td>(Engelberts et al., 2020)</td>
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<td>(..)</td>
<td>Genome</td>
<td>Extremely diverse and flexible, marine endosymbionts with large genome size Functional adaptation and high degree of genomic plasticity due to the large proportion of transposable elements</td>
<td>(Neave et al., 2014, 2016, 2017)</td>
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<tr>
<td><strong>Endozoicomonas</strong></td>
<td>Symbiosis</td>
<td>Endozoicomonas as possible indicator for coral health, Core symbiont of many corals Dominance of <em>Endozoicomonas</em> under coral bleaching</td>
<td>(Neave et al., 2017; Peixoto et al., 2017; Raina et al., 2016; Tandon et al., 2020) (Pogoreutz et al., 2018)</td>
<td></td>
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<tr>
<td><strong>Cyanobacteria</strong></td>
<td><strong>Synechococcales</strong></td>
<td><strong>Thermoanaerobaculum aquaticum</strong></td>
<td>pH</td>
<td>Anaerobic chemoheterotrophs mainly found in freshwater hot springs</td>
<td>(Dedysh et al., 2020; Dedysh and Vilmaz, 2018)</td>
</tr>
<tr>
<td>SB</td>
<td>-</td>
<td>Metabolism and Nutrition</td>
<td><strong>Thermoanaerobacterium hydrothermale</strong></td>
<td>pH</td>
<td>5.5 - 8.5, optimum pH 6.6</td>
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<tr>
<td><strong>Poribacteria</strong></td>
<td>RA, DC, DS2</td>
<td>CV</td>
<td>Metabolism and Nutrition</td>
<td>Autotrophic CO₂-fixation</td>
<td>(Siegl et al., 2011) (Campana et al., 2021)</td>
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<td></td>
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<td></td>
<td>DOM consumption</td>
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<td></td>
<td>Secondary Metabolites</td>
<td>Vitamin B1 synthesis</td>
<td></td>
<td></td>
<td>(Engelberts et al., 2020)</td>
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<tr>
<td></td>
<td>Symbiosis</td>
<td>Possibly ancient symbiont, nearly exclusively found in marine sponges; involved in mediating sponge–microbe interaction</td>
<td></td>
<td></td>
<td>(Siegl et al., 2011)</td>
</tr>
<tr>
<td><strong>Chloroflexi</strong></td>
<td><strong>Dehalococcoidia</strong></td>
<td><strong>SAR202</strong></td>
<td>RA, SB, DC, DS2</td>
<td>CM↓</td>
<td>Genome</td>
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<td>Metabolism and Nutrition</td>
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<tr>
<td><strong>Proteobacteria</strong></td>
<td><strong>Gammaproteob.</strong></td>
<td><strong>Pseudomonadales</strong></td>
<td>SB</td>
<td>-</td>
<td>Metabolism and Nutrition</td>
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<td></td>
<td><strong>SAR86</strong></td>
<td></td>
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<td>Genome</td>
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<tr>
<td>Feature</td>
<td>Method Core</td>
<td>Core</td>
<td>Vent</td>
<td>Putative Functions</td>
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<td><strong>Bacterial symbionts</strong></td>
<td>RA, SB, SM</td>
<td>DC, DS2</td>
<td>TL</td>
<td>Metabolism, Metabolism and Nutrition, Aerobic H₂S-production, Metabolism as major carotenoid pigment produced, Zebatinhsin as major carotenoid pigment produced, Degradation of gelatin and starch, Antifungal activity, biofilm forming, indolic compound production, biosurfactant, and production of the volatile compound 2,4-di-tert-butylphenol (shown in plants)</td>
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<td><strong>Flavobacteria</strong></td>
<td>RA, SB, SM</td>
<td>DS2</td>
<td>TL</td>
<td>Metabolism, Metabolism and Nutrition, Sulfur Metabolism, sulﬁde-oxidizing (SOB) symbiont in the sponge Amphimedon quennestadica, Carbon monoxide oxidation and inorganic phosphate assimilation, Symbiosis, Genomic organization, microbial metabolism, including a versatile nutrient use with large number of transporters, alkyl–alkanolamine-containing proteins, and a CTSR system</td>
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<td><strong>Zothamnion/bacter</strong></td>
<td>RA, SB, SM</td>
<td>DS2</td>
<td>TL</td>
<td>Metabolism, Metabolism and Nutrition, Zothamnion oxidizing nitrous oxide, Nitrite-oxidation, Denitrification and anaerobic ammonium oxidation (anammox), Autotrophic carbon fixation via reductive tricarboxylic acid cycle and cycle</td>
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<td><strong>Metabolites</strong></td>
<td>RA, SB, SM</td>
<td>DC, SM</td>
<td>SV</td>
<td>Metabolism and Nutrition, Metabolism, Metabolism and Nutrition, Metabolism and Nutrition, Metabolism and Nutrition, Metabolism and Nutrition, Metabolism and Nutrition, Metabolism and Nutrition</td>
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<td><strong>Pathogen</strong></td>
<td>RA, SB, SM</td>
<td>DC, SM</td>
<td>SV</td>
<td>Metabolism and Nutrition, Metabolism and Nutrition, Metabolism and Nutrition, Metabolism and Nutrition, Metabolism and Nutrition, Metabolism and Nutrition, Metabolism and Nutrition</td>
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**References**

(Aker et al., 2007; Decleerq et al., 2013; Asker et al., 2007; Decleerq et al., 2013; Lavy et al., 2018; Gauthier et al., 2016; Gauthier et al., 2016; Mohamed et al., 2010; Smitser et al., 2012; Burdardt et al., 2012; Hinsman et al., 2019; Campillay et al., 2012; Caceres et al., 2016; Allen et al., 2018; Burdardt et al., 2012; Zou et al., 2019; Hinsman et al., 2018; Djabie et al., 2008; Georgina et al., 2016; Rubin Blum et al., 2019; Hinsman et al., 2019; Bowden et al., 2006; Zou et al., 2019; Djabie et al., 2008; Georgina et al., 2016; Rubin Blum et al., 2019; Zou et al., 2019; Koler-Costa et al., 2022; Baltari et al., 2022)
<table>
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<tr>
<th>UBA10353</th>
<th>RA, SB, DC, DS2</th>
<th>Mixotroph: Sulfur oxidation and DIC fixation in the dark ocean; genes for taurine utilization, RuBisCO</th>
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</table>
| → now: UBA868|                | Secondary Metabolites Harbour biosynthetic gene cluster for natural products (pederin-type compound “mycalamide”, which has antiviral, apoptotic and protein synthesis inhibiting effects (Dyshlovoy et al., 2012; Hood et al., 2001; Rust et al., 2020)

| Symbiosis | Dominant in vent peripheral sponge Spinularia sp. (Georgieva et al., 2020) |

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<th>Dadabacteria Databacteriales</th>
<th>SB</th>
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<th>Proteobacteria Alphaproteobacteria SAR11</th>
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<th>SAR86</th>
<th>SB, RA</th>
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<th>Bacteroidota Bacteroidia Cryomorphaceae</th>
<th>SB</th>
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<tr>
<th>Metabolism and Nutrition</th>
<th>Some may exhibit photoheterotrophy Cannot utilize carbohydrates and require complex organic compounds for growth (Bowman, 2020; Gómez-Consarnau et al., 2019) (Bowman et al., 2003; Lau et al., 2005; O’Sullivan et al., 2005)</th>
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<th>Genome</th>
<th>enriched in genes that function in cell or colony invasion (Delmont et al., 2015)</th>
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<th>Symbiosis</th>
<th>Mutualistic relationships with Antarctic phytoplankton bloom (Delmont et al., 2015)</th>
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ANY WINNER IN FUTURE OCEAN ACIDIFICATION SCENARIOS?

Different acclimatization traits are likely responsible for the abundance and long-term ecological success of both, the HMA sponge *C. reniformis* and the LMA sponge *S. cunctatrix* at the studied vent system. The HMA sponge showed no significant morphological changes at the vent site and likely profits of a more diverse and richer microbiome. Microbial flexibility has been proposed to be a mechanism for rapid adaptation in corals and sponges to environmental perturbation like OA (Posadas et al., 2022; Ziegler et al., 2019). Restructuring the associated microbiome, possibly influenced by horizontal symbiont transmission, can contribute to survivorship, when functional stability is maintained (Ribes et al., 2016).

Stable symbiotic partnerships of *C. reniformis* with core microbes belonging to *Endozoicomonas*, Chloroflexi (SAR202 and SBR1031), *Bdellovibrio* and Gemmatimonadota, suggest that these taxa performed key metabolic functions for overall holobiont functioning, and likely support resilience to OA due to their versatile and beneficial core functions (Erwin et al., 2015; Webster, 2007). The potential for horizontal gene transfer of the highly abundant *Endozoicomonas* (Neave et al., 2017, 2016), as well as Gemmatimonadota (Zeng et al., 2014) gives rise to the possibility that they were able to acquire genetic traits from OA adapted environmental bacteria (Hentschel et al., 2012; Sobecky and Hazen, 2009), supporting overall holobiont resilience to OA. Furthermore, the introduction of new core microbes at the vent site including Dadabacteriales, Nitrospira and *Constrictibacter* likely compensated for functional losses, which were attributed to control pH associated microbes, such as *Poribacteria*, PAUC34 and Myxococccota.

The core microbiome of *S. cunctatrix* revealed few stable partnerships belonging to Alphaproteobacteria, gammaproteobacterial UBA10353 and Thioglobaceae. Control pH associated taxa included Cryomorphaceae, Dadabacteriales and the alphaproteobacterial clade SAR11. Instead, vent associated symbionts included Cyanobacteria and AqS1. Generally, the LMA microbiome was characterized by a low taxonomic diversity. This likely leads to lower predicted functional redundancy and may be linked to an increased susceptibility to environmental stressors (Girvan et al., 2005; Posadas et al., 2022). In this case, microbial flexibility could be problematic, since restructuring processes may have resulted in loss of essential functions, facilitating pathogen invasions. The higher pumping rates and lower tissue density typical of LMA representatives, provide less protection against the external environment and additionally increase the susceptibility of having more frequent encounters with pathobionts (Posadas et al., 2021). Interestingly, one putative pathogen belonging to Flavobacterialiales was highly abundant at the vent site. Some species belonging to this order were found to be responsible for severe fish diseases causing skin lesions, fin erosion and gill necrosis (Declercq et al., 2013). Three signs that may hint at a microbial dysbiosis were further identified: 1) increased alpha diversities (Luter et al., 2012), 2)
a higher within-group variability in beta diversity, i.e. beta dispersion (Lesser et al., 2016; Pineda et al., 2017) and 3) a shift from sponge enriched microbes towards seawater microbes (Pita et al., 2018; Simister et al., 2012b). Signs of microbial dysbiosis were apparently reflected in morphological changes of S. cunctatrix at the vent site, where the sponges showed reduced body sizes, smaller water canals, very small oscula and overall signs of tissue necrosis. Evidence for tissue necrosis along with increased respiration rates, variation in oxygen consumption rates and mortality were found for the LMA sponge Tethya bergquista after a four-weeks pH 7.6 treatment at 1500 ppm CO₂ (Bates and Bell, 2018). The sponge species Crella incrustans instead, remained in seeming good condition after the same low pH exposures, showing neither negative effects on survival nor signs of disease/necrosis, while maintaining similar respiration rates. Although both of these species are LMA sponges (Moitinho-Silva et al., 2017b), different physiological responses underline species-specificity. Future studies of organismal performance could target the metabolic rates of C. reniformis and S. cunctatrix in situ, to search for similar trends and better assess organismal performance.

Despite showing morphological affections, S. cunctatrix is surviving since many generations at the studied vent site. This implies that despite challenges, the required level of microbial and physiological homeostasis is still maintained. It can be speculated that the sponge-intrinsic immune response of S. cunctatrix sufficiently counteracts invasions of opportunistic microbes and helps maintaining a sufficiently functional microbiome. LMA sponges are considered to maintain their low microbial diversity by efficiently selecting for a consortium of symbionts, while removing non-symbionts through phagocytosis (Lurgi et al., 2019; Posadas et al., 2022). The necessary immune responses might have evolved with the help of different surface receptors, such as NLRs (nucleotide-binding domain, leucine-rich repeat containing), which facilitate the recognition and selection of microbes (von Moltke et al., 2013).

Responses of sponge holobionts to OA seem to be species-specific and, at least to some point, microbially mediated. Many core microbes and symbionts have been identified to change their differential abundances in response to OA. Assigned functions shed light on extremely diverse mechanisms, which are likely involved in microbially mediated acclimation processes. Many microbes likely support host health via nutritional and biogeochemical cycling, including carbon-, nitrogen and sulfur metabolism. Microbes which participate in DOM transformation and degradation, likely play a role in maintaining the productivity of the so called ‘Sponge Loop’ under OA. Other microbes are able to produce secondary metabolites ranging from vitamins, steroid precursor, antimicrobial compound synthesis, as well as compounds involved in osmotic and oxidative stress protection. Many bacterial features with functional convergence were identified, likely supporting the resilience of sponges under OA.
TRENDS OF MICROBIAL FUNCTIONS INVOLVED IN HOLOBIONT RESPONSE TO OCEAN ACIDIFICATION

Elucidating functional roles of lost and newly introduced microbial member taxa can help to estimate relevant metabolic mechanisms in holobiont responses and their adaptation to OA. Repeatedly occurring functions among different sponge-associated microbes indicate key roles of microbial symbiosis in functional redundancy and host stability. Although these functions remain speculative, they present starting points for future investigations using in-depth functional approaches, such as transcriptomics and metabolomics analyses.

Our results suggested, that OA mainly influenced the prevalence of microbial symbionts which play central roles in biogeochemical and nutritional cycling, mainly focussing on carbon-, nitrogen and sulfur-metabolism, as well as vitamin biosynthesis (Table 2.9). Autotrophic CO$_2$ fixation was associated to several sponge associated symbionts, providing their host with the necessary primary metabolism (Cardini et al., 2016; Muscatine and Porter, 1977; Pita et al., 2018). CO$_2$ fixing microbes were introduced into the vent core microbiome of both LMA and HMA sponge species, including cyanobacteria, which fix CO$_2$ via the Calvin–Benson–Bassham cycle (Durall and Lindblad, 2015; Klawonn et al., 2016), Nitrospira, which perform the reductive tricarboxylic acid cycle (Burgsdorf et al., 2022) and the chemoautotrophic UBA10353 (Burgsdorf et al., 2022). Many vent-associated symbionts and core microbiota were further involved in DOM cycling, such as the DOM consuming Endozoicomonas and Nitrospira, as well as the DOM degrading Dadabacteria. Instead, the DOM consuming Poribacteria were associated to the Control site. These trends may suggest that inorganic carbon fixation as well as DOM consumption and transformation are involved in acclimation processes of HMA and LMA holobionts under OA. Nitrogen cycling within the sponge host is highly linked to symbiotic microbiome (Bayer et al., 2008; Fiore et al., 2015; Pita et al., 2018; Webster and Thomas, 2016) and many typical sponge symbionts are enriched in nitrogen metabolism genes (Bayer et al., 2014; Fan et al., 2012; Li et al., 2016). At the CO$_2$ vent site increased abundances were suggested for the nitrate-reducing Candidatus Kaiserbacteria, the nitrate-assimilating Endozoicomonas, and two ammonia-oxidizing symbionts, AqS1 and Nitrospira. Sulfur-oxidising bacteria (SOB) were also found among both sponge species. They oxidize reduced sulfur compounds, including sulfide, elemental sulfur and thiosulfate to obtain energy. At the vent site, the LMA sponge S. cunctatrix showed increased associations with sulfide-oxidizing Gammaproteobacteria, including Thioglobaceae, UBA10353 and AqS1, but also C. reniformis with SAR202. B-vitamin producing phyla were also found among the microbiome of S. cunctatrix and C. reniformis, but in higher diversity and relative abundance in C. reniformis. Results suggested that the potential for B-vitamin biosynthesis had an extensive taxonomical redundancy within the complex sponge microbiome. Many bacterial phyla are considered to contain pathways to
synthesize thiamine, including Acidobacteriota, Actinobacteriota, Bacteroidota, Chloroflexi, Dadabacteria, Gemmatimonadota, Latescibacterota, Nitrospina, Nitrospirota, Poribacteria and Proteobacteria (Engelberts et al., 2020). Functional redundancy seems to be a key feature of holobiont resilience under changing environmental conditions, and specific mechanisms should be proven in future functional studies.

OUTLOOK AND FUTURE STUDIES

This study aimed at shedding light on microbial dynamics of two Mediterranean sponge species in response to OA. Results suggest that both, the HMA sponge C. reniformis and the LMA sponge S. cunctatrix restructured their microbiome via changes in relative abundances and differentially abundant microbes of symbionts (symbiont shuffling), as well as the acquisition or depletion of new ASVs in the vent site (symbiont switching). It would be worth in upcoming studies to estimate the weight of horizontally versus vertically transmitted microbes taking part of the core microbiomes, and under control and OA scenarios. This could be empirically approached analysing incurrent and excurrent filtering fluxes and larval stages, via metabarcoding and FISH (Fluorescent in situ Hybridization) techniques (Moter and Göbel, 2000).

Discussed metabolic functions, which are putatively performed by sponge-associated microbiomes are estimates and speculative interpretations based on genetic information of cultivated microbial taxa or single-cell genomic analyses. Further validation will be required to improve mechanistic understanding of holobiont components. Which genetic and/or metabolic pathways become activated, up and down regulated in host cells and the microbial compartment in sponges coming from control sites and in vent systems, can be assessed with meta-transcriptomics studies. And this can inform about the biochemical potential of the sponge microbiome, and reveal functional shifts under OA. It can also reflect the expression levels of certain immune response genes that affect microbiome structure and host-microbe interactions (Posadas et al., 2022).

It is widely assumed that symbiotic microorganisms exchange molecules as nutrients, vitamins and allelochemicals with their hosts, but this remains still unstudied in acidified systems. Hence, testing these trophic biochemical relationships under OA conditions, through stable isotope signatures and other isotopic techniques will be crucial to further understand host-microbe interactions. Additionally, and fundamentally, it will be of key significance to compare sponge metabolic patterns between vent and control sites, and to quantify key metabolites which might be involved in acclimatization processes, via untargeted and targeted metabolomics profiling.
SUPPLEMENTARY MATERIAL

RAREFACTION CURVES

For each sampling depth the curves plateaued, indicating that additional sampling efforts would not result in changes in abundance and evenness of microbial taxa per sample. The average observed features are shown for each sequenced sample, including the boxplots—minimum, median and maximum (Figure S.1).

Figure S.1: Alpha Rarefaction curve.

ALPHA DIVERSITY

Figure S.2: Alpha diversity of *C. reniformis* (HMA), *S. cunctatrix* (LMA) and seawater. Diversity metrics were calculated for each species and seawater samples. Boxplots show range (whiskers), median (bold line), and interquartile range (box height).
Figure 5.3: Heatmap of the hierarchical clustering at ASV level from Gneiss linear regression of sponge and seawater samples from vent and control sites. ASVs which were focus of gneiss analyses are present in the balance y0. The vertical bar indicates in light red numerators and in dark red denominators used to calculate microbial balances.
Figure S.4: Bdellovibrio-to-SAR202 balance showing differential abundances in *C. reniformis* from vent and control site.

Figure S.5: Endozoicomonas-to-Burkholderiales balance showing differential abundances in *C. reniformis* from vent and control site.
**Endozoicomonas**

**Chloroflexus**

Figure S.6: Endozoicomonas-to-uncultured_Chloroflexus balance showing differential abundances in *C. reniformis* from vent and control site.

**UBA10353**

**Zeaxanthinibacter**

Figure S.7: UBA10353-to-Zeaxanthinibacter balance showing differential abundances in *S. cunctatrix* from vent and control site.
Figure S.8: UBA10353-to-Pseudomonadales balance showing differential abundances in *S. cunctatrix* from vent and control site.

Figure S.9: SAR11-to-Pseudomonadales differential abundance in *S. cunctatrix* from vent and control site.
DESEQ2 - DIFFERENTIAL ABUNDANCE ANALYSES

The DeSeq2 method was performed to complement and confirm differentially abundant taxa found to be associated with the control or vent site for each species. A total of 9 and 18 unique ASVs were differentially abundant in *C. reniformis* and *S. cunctatrix*, respectively. Increases in log ratios were driven by association to the vent site.

DeSeq2 results revealed similar trends for differentially abundant taxa at the vent site, like beforehand predicted using the tool Songbird. In the HMA sponge, two features belonging to the order Pseudomonadales, genus *Endozoicomonas* and an uncultured feature were associated to the vent conditions, while the gammaproteobacterial K189A clade was correlated to control sample (Figure S.10). Further features associated to control pH conditions were SAR202, Poribacteria and Nitrosococcales - AqS1. In the LMA sponge, the genus *Nitrospira* was strikingly more abundant at the vent site (Figure S.11). Also, genus *Zeaxanthinibacter* (order Flavobacterales) and Nitrosococcales showed increases at the vent site, confirming results obtained from Songbird.

Negative log ratios were found for alphaproteobacterial features, suggesting their association with the control site. Contradictive results were observed for the gammaproteobacterial UBA10353 which according to Songbird and Deicode analyses showed stronger association to control samples, but DeSeq2 suggest a strong association to the vent site.

**Figure S.10:** Differentially abundant taxa for *C. reniformis* calculated with DeSeq2 for the taxonomical levels phylum, class, order and genus. Circles represent differentially abundant taxa at calculated log-ratios. Features with positive log-ratios were more abundant at the vent site, whereas negative log fold changes represent a higher association to the control site.
Figure S.11: Differentially abundant taxa for *S. cunctatrix* calculated with DeSeq2 for the taxonomical levels phylum, class, order and genus. Circles represent differentially abundant taxa at calculated log-ratios. Features with positive log-ratios were more abundant at the vent site, whereas negative log fold changes represent a higher association to the control site.
Table S.1: Stable core taxa and core functions of the microbiome associated to *C. reniformis*. Putative core functions and possible interpretation were based on Pita et al., 2018.

<table>
<thead>
<tr>
<th>Core Microbe =</th>
<th>Putative Core Function</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Endozoi-comonas</em></td>
<td>Participate in host-associated protein and carbohydrate transport and cycling, production of amino acids (Neave et al., 2017, 2016) Nitrate assimilation (Neave et al., 2014) DOM consumption (Campana et al., 2021) Mobile genetic elements and transposases (Neave et al., 2017, 2016)</td>
<td>Utilization of environmental and host-derived nutrients (Kamke et al., 2013; Slaby et al., 2017) Increased levels of horizontal gene transfer (Fan et al., 2012; Thomas et al., 2010)</td>
</tr>
<tr>
<td>Chloroflexi: SBR1031, A4b</td>
<td>Nitrogen metabolism: anaerobic (Yamada et al., 2006) nitrifying (Li et al., 2020) symbionts Degradation of polycyclic aromatic hydrocarbons (Wu et al., 2022)</td>
<td>Utilization of environmental and host-derived nutrients (Kamke et al., 2013; Slaby et al., 2017)</td>
</tr>
<tr>
<td>Chloroflexi: SAR202</td>
<td>Nitrogen metabolism: nitrate reduction (Thrash et al., 2017) Degradation of complex carbohydrates (Thrash et al., 2017), including polycyclic aromatic hydrocarbons (Colatriano et al., 2018; Wang et al., 2020) Sulfite-oxidizer (Mehrshad et al., 2018)</td>
<td>Utilization of environmental and host-derived nutrients (Kamke et al., 2013; Slaby et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>Symbiont in sponge extracellular matrix with CRISPR-Cas systems, eukaryote-like repeat proteins, and secondary metabolite gene clusters (Bayer et al., 2018)</td>
<td>Defense against viruses/phages (Horn et al., 2016; Slaby et al., 2017; Thomas et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Degradation of labile and recalcitrant DOM (Bayer et al., 2018; Landry et al., 2017) DOM consumption (Campana et al., 2021)</td>
<td>Nutrient Supply &amp; DOM recycling for Sponge Loop (Bayer et al., 2018; Landry et al., 2017)</td>
</tr>
<tr>
<td>Bdellovibrio nota: <em>Bdellovibrio</em></td>
<td>Prey non-specifically on numerous bacterial gram-negative pathogens (Cavallo et al., 2021; Harini et al., 2013; Jurkevitch, 2012; Koval et al., 2013; Markelova, 2010; Starr and Baigent, 1966)</td>
<td>'Amphibiotic' biocontrol agent: probiotic &amp; 'living antibiotic' (Cavallo et al., 2021; Harini et al., 2013; Jurkevitch, 2012; Koval et al., 2013; Markelova, 2010; Starr and Baigent, 1966)</td>
</tr>
<tr>
<td>Gemmatim onadota: BD2-11 (terrestrial)</td>
<td>Photosynthetic gene acquisition via horizontal gene transfer (Zeng et al., 2014)</td>
<td>Increased levels of horizontal gene transfer (Fan et al., 2012; Thomas et al., 2010)</td>
</tr>
</tbody>
</table>
Table S.2: Dynamic core taxa and core functions of the microbiome associated to *C. reniformis*. Putative core functions and possible interpretation were based on Pita et al., 2018.

<table>
<thead>
<tr>
<th>Core Microbe</th>
<th>Putative Core Function</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gammaproteob.</td>
<td>Very diverse group of sponge symbionts, not generalizable.</td>
<td>-</td>
</tr>
<tr>
<td>Nitrospirota: <em>Nitrospira</em></td>
<td>Nitrogen Cycling: nitrite-oxidation (Burgsdorf et al., 2022; Hoffmann et al., 2009; Simister et al., 2012b), ammonia-oxidation (Mohamed et al., 2010; Simister et al., 2012b) Carbon fixation (Burgsdorf et al., 2022) and DOM consumption (Campana et al., 2021)</td>
<td>Utilization of environmental and host-derived nutrients (Kamke et al., 2013; Slaby et al., 2017; Webster and Thomas, 2016)</td>
</tr>
<tr>
<td>Alphaproteob.</td>
<td>Very diverse group of sponge symbionts, not generalizable.</td>
<td>-</td>
</tr>
<tr>
<td>Alphaproteob.: <em>Constrictibacter</em></td>
<td>Carbon metabolism (Yamada et al., 2011)</td>
<td>Utilization of environmental and host-derived nutrients (Kamke et al., 2013; Slaby et al., 2017)</td>
</tr>
<tr>
<td>Dabadacteria: Dabadacteriales</td>
<td>Photoheterotroph (Graham and Tully, 2021) DOM-degradation peptidoglycan and phospholipids (Graham and Tully, 2021) Vitamin B1 synthesis (Engelberts et al., 2020) Steroid precursor biosynthesis $\rightarrow$ Secondary metabolism95</td>
<td>Vitamins can be utilized by the sponge host; Microbe-microbe interaction, defense of the holobiont (Fan et al., 2012; Fiore et al., 2015; Thomas et al., 2010)</td>
</tr>
<tr>
<td>Poribacteria</td>
<td>Carbon metabolism: autotrophic CO2-fixation Vitamin B1 synthesis (Engelberts et al., 2020) DOM consumption (Campana et al., 2021)</td>
<td>Utilization of environmental and host-derived nutrients (Kamke et al., 2013; Slaby et al., 2017) Vitamins can be utilized by the sponge host (Thomas et al., 2010) Mediation of sponge–microbe interactions (Siegl et al., 2011)</td>
</tr>
<tr>
<td>PAUC34</td>
<td>Potential for degradation of sponge- and algae-derived carbohydrates (Astudillo-García et al., 2018) capacities for complex carbohydrate degradation (Thrash et al., 2017)</td>
<td>Utilization of environmental and host-derived nutrients (Kamke et al., 2013; Slaby et al., 2017)</td>
</tr>
<tr>
<td>Acidobacteria: Vicinamibacteria</td>
<td>Chemoorganoheterotrophs (Huber and Overmann, 2019)</td>
<td>-</td>
</tr>
<tr>
<td>Myxococcota</td>
<td>Secondary Metabolites (Gemperlein et al., 2018; Herrmann et al., 2017), production of antibiotics (Schäberle et al., 2014)</td>
<td>Microbe-microbe interaction, defense of the holobiont (Fan et al., 2012; Fiore et al., 2015; Thomas et al., 2010)</td>
</tr>
</tbody>
</table>
Table S.3: Stable core taxa and core functions of the microbiome associated to *S. cunctatrix*. Putative core functions and possible interpretation were based on Pita et al., 2018.

### = *S. cunctatrix*: Stable Core Microbiome

<table>
<thead>
<tr>
<th>Core Microbe</th>
<th>Putative Core Function</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gammaproteob.: UBA10353</td>
<td>Carbon and Sulfur Metabolism: Chemoautotrophic carbon fixation in sponges energized by thiosulfate oxidation (Burgsdorf et al., 2022), (Baltar et al., 2022)</td>
<td>Utilization of environmental and host-derived nutrients (Kamke et al., 2013; Slaby et al., 2017; Webster and Thomas, 2016)</td>
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<tr>
<td></td>
<td>Secondary Metabolism: biosynthetic gene cluster for natural products (pederin-type compound “mycalamide”) (Dyshlovoy et al., 2012, Rust et al., 2020)</td>
<td>Defense strategies: antiviral, apoptotic (Hood et al., 2001) and protein synthesis inhibiting effects (Dyshlovoy et al., 2012, Rust et al., 2020)</td>
</tr>
<tr>
<td>Alphaproteob.</td>
<td>Very diverse group of sponge symbionts, not generalizable.</td>
<td>-</td>
</tr>
<tr>
<td>Thioglobaceae</td>
<td>Sulfur Metabolism: sulfide and/or methane oxidation (Ansorge et al., 2020, Rubin-Blum et al., 2019; Zhou et al., 2019)</td>
<td>Chemosynthetic symbioses (Hestetun et al., 2016; Rubin-Blum et al., 2019; Zhou et al., 2019)</td>
</tr>
</tbody>
</table>

Table S.4: New core taxa introduced at vent site. Best hit taxonomical annotations of identified core taxa are listed with corresponding feature IDs in decreasing order of relative core abundance (up to down). Putative core functions and possible interpretation were based on Pita et al., 2018.

### ⇧ *S. cunctatrix*: New core taxa at the CO2 vent

<table>
<thead>
<tr>
<th>Core Microbe ⇧</th>
<th>Putative Core Function</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavobacteriales: <em>Zeaxanthinibacter</em></td>
<td>Sulfur-Metabolism (H2S production) (Declercq et al., 2013) ‘Zeaxanthin’ as major carotenoid pigment (Asker et al., 2007) Degradation of gelatin and starch (Asker et al., 2007) Fish Pathogen: Tissue necrosis via degradation of complex acidic polysaccharides of connective tissue (Declercq et al., 2013) Secondary Metabolites with antifungal activity (Sang and Kim, 2012)</td>
<td>Utilization of environmental and host-derived nutrients (Kamke et al., 2013; Slaby et al., 2017; Webster and Thomas, 2016) Possible correlation with tissue necrosis (Declercq et al., 2013)</td>
</tr>
<tr>
<td>Gammaproteob.</td>
<td>Very diverse group of sponge symbionts, not generalizable.</td>
<td>-</td>
</tr>
<tr>
<td>Flavobacteriales: <em>Cyclobacteriaceae</em></td>
<td>DOM consumption (Campana et al., 2021)</td>
<td>Utilization of environmental and host-derived nutrients (Kamke et al., 2013; Slaby et al., 2017; Webster and Thomas, 2016)</td>
</tr>
<tr>
<td>Cyanobacteria: <em>Cyanobium</em></td>
<td>Autotrophic carbon-fixation (Durall and Lindblad, 2015; Klawonn et al., 2016) Nitrogen Fixation (Bednarz et al., 2017)</td>
<td>Utilization of environmental and host-derived nutrients (Kamke et al., 2013; Slaby et al., 2017)</td>
</tr>
<tr>
<td>Acidobacteriota: <em>Themoanabaculaceae</em></td>
<td>Anaerobic chemoheterotrophs (Dedysh et al., 2020; Dedysh and Yilmaz, 2018)</td>
<td>Utilization of environmental and host-derived nutrients (Kamke et al., 2013; Slaby et al., 2017)</td>
</tr>
</tbody>
</table>
Raw reads and the ASV table are provided via the following online repository link - CHAPTER II:
https://drive.google.com/drive/folders/1uRAx6tEs5SWHEJNdxsIgj6S_6wcdAVO?usp=share_link.

The table with the file name “ASV_table_sequences_raw.xlsx” reports raw genetic sequences and assigned taxonomies for 1676 feature IDs (ASVs). The relative abundance of ASVs per sample is listed in the table “Relative_Abundance_per_Sample_rarefied.xlsx” for a total of 385 rarefied ASVs. Sample metadata are provided as a text file with the filename “sample-metadata.txt”. After article publication, all fastq files will be available on the NCBI repository under the following link: https://www.ncbi.nlm.nih.gov/sra/PRJNA926125.
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CHAPTER III

METABOLIC SHIFTS IN SPONGE HOLOBIONTS IN RESPONSE TO OCEAN ACIDIFICATION
ABSTRACT

Ocean acidification (OA), driven by increasing human CO$_2$ emissions represents an emerging threat to marine species and ecosystems. Changes in environmental conditions can modulate biochemical pathways and affect individual metabolite compositions. Marine sponges represent an ideal system to study the dynamics of host-symbiont interactions, and organismal adaptation related to metabolite production, during natural or anthropogenic-derived fluctuations in marine ecosystems. Metabolic profiles of sponges inhabiting under the influence of a volcanic CO$_2$ vent with naturally lowered pH conditions (7.6 – 7.88), were compared to those of specimens living in a control site with normal pH (8.2). NMR based metabolomics were performed in two Mediterranean sponge species – the high-microbial (HMA) sponge Chondrosia reniformis and the low microbial abundance (LMA) sponge Spirastrella cunctatrix. First one- and two-dimensional NMR spectra were interpreted capturing the composition of crude polar extracts. Most compounds identified were primary metabolites, including amino acids and their derivatives, as well as metabolites of common central metabolism. Metabolites identified exclusively in the HMA sponge included distinct peaks corresponding to histamine and imidazole, while the LMA sponge spectra showed strong signals for trigonelline, allantoin and betaine. The application of multivariate statistics (OPLS-DA) predicted discriminant NMR variables from both sponge species from vent and control sites. No significant metabolite shifts were detected for in C. reniformis. Instead, S. cunctatrix from the vent site showed decreased levels of the osmoregulatory metabolites taurine and trigonelline, and increased concentrations of the osmolyte betaine. Moreover, metabolites involved in the energy metabolism, such as creatine and AMP, exhibited decreased relative abundances, as well as the oxidative stress marker allantoin. In lieu, increased levels of amino acids, lipids and choline were detected at the vent site.

Metabolic shifts in the LMA sponge S. cunctatrix suggest, that OA impacted several pathways, including osmoregulation, energy metabolism, anaerobic metabolism, lipid metabolism and oxidative stress responses. The lack of significant metabolite shifts in the HMA sponge C. reniformis suggests maintenance of metabolic homeostasis and support species-specific responses to OA.

Keywords: Ocean Acidification, Metabolomics, Porifera, HMA and LMA sponges, Multivariate Analysis, OPLS-DA, Climate Change, CO$_2$ vent,
INTRODUCTION

OCEAN ACIDIFICATION

Ocean acidification (OA), driven by increases in human CO₂ emissions represents an increasing threat to marine species and ecosystems (Doney et al., 2009). Sea surface pH has declined by about 0.1 pH units since pre-industrial times (Bindoff et al., 2007). Depending on the magnitude of ongoing greenhouse gas emissions from human fossil fuel combustion and deforestation, a drop in pH of 0.4 - 0.5 units is predicted for 2100 (Caldeira and Wickett, 2003; Doney et al., 2009; Doney and Schimel, 2007; Gattuso and Hansson, 2011). This predicted drop from pH 8.1 to approximately pH 7.7, is the result of an increased dissolution of atmospheric CO₂ into the oceans and changes the carbonate chemistry. Upon absorption of CO₂ by the seawater, carbonic acid (H₂CO₃) is formed, which dissociates into free H⁺ and bicarbonate ions (HCO₃⁻). Bicarbonate can further break into carbonate and H⁺. The increasing proton concentration is finally responsible for the acidification phenomenon due to drop in pH (Figure 3.1). A great number of research publications reported negative impacts on numerous calcifying organisms, such as reef-building corals (e.g. Andersson and Gledhill, 2012, 2013; Chan and Connolly, 2013). Calcification becomes more difficult in an increasingly acidified environment, because carbonate concentrations decrease, a key structural component for calcitic shell formation, while more bicarbonate and carbonic acid is produced (Andersson and Gledhill, 2013; Doney et al., 2009).

Figure 3.1: Carbonate chemistry and the effects of dissolved CO₂ in seawater responsible for ocean acidification (CSU, 2019).
Non-calcifying reef-building organisms, such as sponges, also experience challenges in an acidified environment. Sponges lack a complex, protective body structure and their interior is only separated by one cell layer from the external environment (Bergquist, 1978). This enhances the risk of H⁺ ions passing the outer membrane and increasing intracellular acidity, causing acid-base imbalances. The maintenance of intracellular pH values and chemistry to protect cellular and metabolic processes (Pörtner and Farrell, 2008) are crucial and likely require mechanisms for osmo-/iono-regulation (Dissanayake et al., 2010). Mechanistically it is poorly understood how sponges cope with acidification stress, and it seems that not every sponge species is resilient to OA at the same level (Bell et al., 2018a).

**METABOLIC PROCESSES UNDER OCEAN ACIDIFICATION**

Changes in environmental conditions can modulate biochemical pathways that underlie individual phenotypes, finally affecting individual metabolite compositions (Bundy et al., 2009; Michal and Schomburg, 1999; Wagner et al., 2013). By definition, metabolites are low molecular weight organic compounds within a cell, tissue or biofluid, that derive as products or intermediate molecules of metabolism, such as e.g. glucose, acetate, alanine and lipids (Griffiths, 2007; Harrigan and Goodacre, 2003; Lindon et al., 2006). The analyses of metabolites, integrated with physiological responses of marine invertebrates to stress conditions, is a promising strategy to better predict an organism’s performance and reveal biomarkers for climate change studies. Analytical tools suitable to rapidly measure changes in invertebrate performance in the face of acidification can be based on using several ‘omics’ approaches, including transcriptomics, proteomics, and metabolomics. One recent study analyzed the metabolomic and transcriptomic profiles of oyster larvae in response to experimental OA conditions. It could be shown that stress-induced impairment of larval shell formation were accompanied by a significant alteration of energy metabolic processes, including amino acid oxidation, glycolysis, pentose phosphate pathway and fatty acid metabolism (Liu et al., 2020). Another study applied a non-targeted metabolomics approach on corals that had been exposed to near future climate change conditions. Corals altered their carbohydrate composition, cell structural lipids and signaling compounds in response to OA and elevated temperatures (Sogin et al., 2016). To unravel physiological responses of the Dungeness crab on a molecular level, Trigg et al. (2019) performed untargeted metabolomics on individual crab juveniles. They found 94 metabolites responding in a condition-specific manner to low pH and low oxygen exposures. The increasing number of publications aiming at answering ecological challenges applying metabolomics studies are revealing promising insights into molecular responses of marine species to climate change stressors. However, these studies are for the most part conducted with animals exposed to artificially induced stress-conditions in aquaria (e.g., Ellis et al., 2014; Gaubert et al., 2020; Liu et al., 2020; Sogin et al., 2016; Trigg et al., 2019),
and more studies of long-term adapted species (e.g., (Kumar et al., 2022, 2018) are required to better capture the natural picture of molecular responses. Metabolomics studies of adapted marine species inhabiting natural CO\textsubscript{2} vent sites are very scarce, although these ecosystems are highly suitable to predict long-term effects of OA on the physiology and biochemistry of an organism (Fabricius et al., 2011; Hall-Spencer et al., 2008). One recent study assessed the possible effects of OA on the metabolic potential of sponges and their symbionts applying metagenomic analyses techniques (Botté et al., 2019; Proksch et al., 2002; Zhang et al., 2005). Several metabolic features were suggested to contribute to an enhanced tolerance of the sponge symbionts, and possibly their host to better withstand OA. Impacted pathways included the transport of carbon and host-derived compounds, as well as nitrogen and sulfur metabolism (Botté et al., 2019).

THE SPONGE METABOLOME

Marine sponges represent an ideal system to study the dynamics of host-symbiont interactions and their role in organismal adaptation to changing environmental conditions (Hentschel et al., 2012; Van Soest et al., 2012). They form intimate associations with diverse microorganisms (Moitinho-Silva et al., 2017a) and can be classified as high-microbial-abundance (HMA) and low-microbial abundance (LMA) species, depending on their microbial load (Erwin et al., 2015). The largest repertoire of metabolites in eukaryotic organisms derive from associated microorganisms, such as viruses, archaea, bacteria, dinoflagellates and fungi, which are often producers and/or consumers of metabolites and synergistically impact the metabolic pool of their hosts (Della Sala et al., 2014; Hentschel et al., 2012; McHardy et al., 2013; Morita and W. Schmidt, 2018; Trindade-Silva et al., 2012; Zhang et al., 2022). A remarkable metabolite diversity was uncovered among sponges, including terpenes, sterols, cyclic peptides, unusual nucleosides, alkaloids, fatty acids, peroxides, and amino acid derivatives (Blunt et al., 2015; Faulkner, 2002; Perdikaris et al., 2013; Rohde et al., 2012).

Marine microorganisms are more metabolically diverse than their terrestrial counterparts and comprise more of ancient lineages, rendering them of interest in drug discovery and natural product research (Zhang et al., 2005). Sponges harbor highly diverse symbiotic communities, producing numerous bioactive compounds with biomedical application (Anjum et al., 2016; Proksch et al., 2002; Waters et al., 2014). Therapeutic potential have been ascribed to these natural products, due to their anti-inflammatory (Lee et al., 2019), anticancer (Curman et al., 2001; Heidary Jamebozorgi et al., 2019), antimicrobial (Heidary Jamebozorgi et al., 2019; Radnaeva et al., 2020; Viegelmann et al., 2014), anti-atherosclerotic (Mohamad et al., 2017) and antiviral properties (Souza et al., 2007).
Some direct ecological benefits for sponges are mediated by microbial metabolites. For instance, toxic defensive molecules protect sponges from predators and repel or even kill fish (Kashman et al., 1980; Pawlik et al., 1995). Other metabolites, such as lysophospholipids, may regulate cell division and differentiation, acting as signaling molecules during embryogenesis and morphogenesis, while also providing a lipid reserve (Ivanisevic et al., 2011). Metabolites with antifouling activities, prevent other organisms from settling or overgrowing the sponge (Aguila-Ramírez et al., 2014; Satheesh et al., 2016; Thompson et al., 1985). However, in comparison to therapeutic or pharmacological applications, relatively little is known about biological benefits provided by symbiont-produced compounds, especially in the context of climate change stress and organismal homeostasis.

NMR BASED METABOLOMICS

Nuclear magnetic resonance was first described and measured in gas molecular beams by Isidor Rabi (1938), and then expanded to liquids and solids by Felix Bloch and Edward Mills Purcell, for which they shared the Nobel Prize in Physics in 1952. Later, Lauterbur (1973) reconstructed a density map, which visualized proton spins derived from NMR and demonstrated two-dimensional imaging, the scientific principle used later for magnetic resonance imaging (MRI). Mansfield and Grannell then described the Fourier relationship between the spin density and the NMR signal in the presence of a magnetic field gradient (Callaghan, 1993; Mansfield and Grannell, 1975). Among other advances, these milestones in the development of NMR spectroscopy made the structural analyses of chemical compounds and metabolomics studies that we use today possible.

The basic physicochemical principle to obtain structural information about molecules using NMR is schematically visualized in Figure 3.2. Key of this technique is the application of an external radiofrequency radiation which interacts with atomic nuclei, resulting in a net exchange of energy and leading to a change in their nuclear spins. The nuclear spin is defined by a quantic number (l) which depends on the isotope. NMR spectroscopy is only able to detect atomic nuclei with $I \neq 0$, i.e. NMR-active nuclei which behave like a magnetic dipole, include $^1$H, $^2$H, $^{13}$C and $^{15}$N. When applying an external magnetic field, the spins of nuclei align and adopt two different orientations within the field, either parallel (lowest energy level) or antiparallel (highest energy level). Magnetic resonance is achieved when nuclei are irradiated with the applied radiofrequency, causing changes in the orientation of nuclear spins (i.e. transitions between energy levels). Within the magnetic field, nuclei are not statically aligned, but rather move like a spinning top in the so called ‘precession movement’ around an axis parallel to the magnetic field. The frequency of this precession movement is called ‘Larmor frequency’. By applying magnetic pulses containing frequencies close to the Larmor frequency, the resonance of magnetized nuclear spins is reached. After this pulse, a
relaxation process takes place where the nuclear spins gradually recover and flip back into their natural state where, depending on the molecular structure and neighbouring atoms, each spin has a unique thermal equilibrium. As a consequence of this relaxation, energy is emitted as radiofrequency, producing a characteristic signal called free induction decay (FID) which is registered by the detector. This FID is subsequently transformed into a plot of intensities (by Fourier transformation) versus frequencies known as an NMR spectrum (Atta-Ur-Rahman, 2012; Carreras, 2021; Diehl et al., 2013; Friebolin and Becconsall, 2005; Larive et al., 2014).

![Figure 3.2: Principles of NMR metabolomics.](image)

1) Biological samples are extracted yielding a complex metabolite mixture, which are transferred into an NMR tube. 2) During NMR acquisition a strong homogenous magnetic field aligns the nuclear spins of the atoms present in the sample by using the same resonance frequency of the nuclei (Larmor Frequency). The nuclear spins are magnetized and resonate. After the magnetic pulse, spins ‘flip back’ to their natural state (thermal equilibrium) and their relaxation times yield a measurable signal, the free induction decay (FID). 3) The FID is translated via Fourier-Transformation (FT) into frequencies (Hz) and yield the final metabolomics spectrum, which can be processed and analysed for metabolite identification and abundance analyses.

In natural chemical extracts, a great number of signals is recorded simultaneously from all present metabolites and yield a very complex spectrum. For qualitative metabolite identification, peaks (NMR variables) are compared to existing databases according to their chemical shifts and coupling constants (multiplicity, i.e. the form of a peak). However, NMR signals yield not only qualitative information, but peak areas and height give information about the quantity of each compound (Z.-F. Wang et al., 2021). By integrating the areas under the signals of an NMR spectrum, relative concentrations of compounds with magnetically different nuclei can be obtained. For the determination of absolute concentrations the addition of a standard with known concentration is required (Paruzzo et al., 2020). The hallmark of NMR spectra is their outstanding reproducibility and specificity for metabolites across different spectrometers and users (Bingol and Brüschweiler,
Over the past years NMR metabolomics databases and repositories have undergone significant expansions, facilitating the identification of known compounds (Bingol et al., 2016). However, the identification of ‘unknown’ metabolites is still one of the major challenges in the metabolomics field.

A commonly used approach in NMR-based metabolomics is the analysis of one dimensional (1D) $^1$H NMR spectra, which rely on the information obtained from hydrogen atoms of target metabolites. In highly complex mixtures, where many peaks tend to overlap, spectral resolution is substantially improved when adding two-dimensional (2D) NMR experiments (Bingol and Brüschweiler, 2014; Clendinen et al., 2014). In 2D experiments, spin magnetization is transferred between different nuclear spins and reveal ‘cross-peaks’ when plotting their recorded spectra against two frequency axes (Bingol et al., 2016). This reduces greatly overlapping areas of recorded peaks and facilitates metabolite identification and structure validation. The most commonly used 2D experiment is the $^{12}$C-$^1$H heteronuclear single quantum coherence spectroscopy (HSQC) which shows the correlation between chemical shifts of $^1$H nuclei with their directly bound $^{13}$C nuclei. Further, the 2D $^1$H-$^1$H total correlation spectroscopy (TOCSY) experiment is widely used for structure validation, providing chemical shifts of all $^1$H spin systems within a molecule or spin system (Guennec et al., 2014; Hansen et al., 2021; Martineau et al., 2020).

The analysis of metabolic shifts of reef building organisms in changing seawater conditions is of particular interest for marine biology scientists to identify biomarkers for climate-change related stress responses (Sogin et al., 2014). Sponge metabolomics under climate change stressors such as OA have received scarce attention, and little is known about molecular mechanisms taking place in organismal adaptation or stress responses. This chapter aims at capturing for the first time the polar fraction of metabolite profiles extracted from the HMA sponge *C. reniformis* and the LMA sponge *S. cunctatrix* collected from a CO$_2$ vent with lowered pH values and a control site with “normal” pH. The main scopes are to present the first NMR spectra recorded for these species, estimate their metabolome complexities, identify known metabolites, and shed light on relative abundance shifts of identified metabolites for both species in response to OA.

**STATEMENT OF CONTRIBUTION**

Metabolomics analyses were performed in collaboration with Dr. Marianna Carbone, Dr. Debora Paris and Dr. Andrea Motta at the Institute of Biomolecular Chemistry at the National Research Council (ICB-CNR, Pozzuoli, Italy), where these colleagues supervised the settings of the NMR spectrometer and enabled me to acquire NMR spectra. Dr. Debora Paris guided the multivariate statistics analyses and metabolite identification.
**MATERIAL & METHODS**

**SPONGE SAMPLING**

Sponge samples (n = 40) of two target species – *Chondrosia reniformis* (HMA) and *Spirastrella cunctatrix* (LMA), were collected in July 2021 by scuba diving at 2-4 m depth in individual zip bags, and kept in a cooler on board. Sponge species identification was based on their morphology and DNA barcoding for taxonomical confirmation (not shown). To minimize variability, two sites with similar environmental parameters of depth, temperature and orography were selected. Sampling sites were located around Ischia Island in the Gulf of Naples (Italy) and consisted of two semi-submerged caves: the acidified cave Grotta del Mago (40°42′41.87″N, 13°57′51.06″E) presenting CO₂ bubbling and pH values of 7.6 – 7.88; and a control pH cave Punta Vico (40°45′32.28″N, 13°52′55.38″E) with pH values of 8.04 – 8.05 (Figure 3.3; Teixidó et al., 2020). After sampling, sponge individuals were transported in coolers to the laboratory, where sub-samples (∼10 cm³) were rinsed with sterile seawater, snap-frozen in liquid nitrogen and stored at -20°C until chemical extraction.

![Figure 3.3: Sampling Sites off Ischia Island (Italy).](image)

**Chemical Extraction of Sponge Samples According to Bligh and Dyer**

The extraction protocol according to Bligh-Dyer (Figure 3.4) was modified from a multi-omics compatible protocol provided by Agilent (Blackwell et al., 2013). Lyophilized sponge samples were grinded with liquid nitrogen using a pestle and mortar. After transferring 0.5 g of sponge powder into chemically resistant 50 mL Falcon tubes, 10 mL of MeOH (methanol) and 4 mL H₂O (MilliQ) were added. Samples were vortexed and sonicated for 1 minute at 600 W. Then, 5 mL of CHCl₃ (chloroform) were added and samples were vortexed and incubated in an ice-cold sonication bath for 5 minutes. 5 mL of H₂O were added and vortexing and incubation in the sonication bath were repeated. After the final addition of 5 mL of CHCl₃, final vortexing and incubation in the sonication bath, the Bligh-Dyer ratio of MeOH:CHCl₃:H₂O (2:2:1.8) was reached. To facilitate phase separation of the solvent system, samples were centrifuged (Centurion Scientific K3 series) at 4000 rpm for 20 minutes. A further incubation of 10 min at room temperature additionally supported a clean phase separation of the polar solvents MeOH/H₂O, the intermediate protein disk and the lower, apolar phase of CHCl₃ (see Figure 3.4). Carefully, 15 mL of the upper phase and 8 mL of the lower phase were transferred into a glass balloon and dried down on a Rotavapor. Extracts were transferred into pre-weighed glass vials and dried under nitrogen stream. Samples were stored at -20°C until analyses. For each sponge species a total of 10 replicates per control and vent site were extracted for downstream analyses. All chemical extractions were performed at Gavagnin-Carbone Lab at the Institute of Biomolecular Chemistry at the National Research Council (ICB-CNR, Pozzuoli, Italy).

**Figure 3.4:** Schematic overview of the Bligh-Dyer extraction protocol performed on lyophilized sponge samples.
NMR based metabolomics analyses of sponge chemical extracts were performed according to the workflow schematically visualized in Figure 3.5. After first acquiring the chemical fingerprints of present molecules by recording NMR spectra, which are used for the identification of metabolite typical chemical shifts. This allows to assign metabolites and describe the chemical composition of sponge extracts.

Since high resolution NMR spectra of crude extracts are highly complex, visual inspection would not be discriminative enough to allow the discrimination of specific metabolites. The application of multivariate statistics helps describing the metabolic compositions and reveal trends across sample groups (Lindon et al., 2001). As a necessary first step of investigation, multivariate data analysis was applied on selected NMR variables to reveal an approximation of the most discriminating variables, i.e. metabolites, and their correlation to sponge extracts derived from sponges inhabiting CO\textsubscript{2} vent sites versus control pH sites.

Finally, the predicted up- or down-regulated metabolites in sponge extracts were evaluated using a univariate t-test, to quantitatively compare relative metabolite concentrations across vent and control site for both sponge species.

**Figure 3.5: Metabolomics workflow.**
**NMR Sample Preparation**

Each polar extract was resuspended in 630 µL phosphate-buffered saline (PBS) buffer solution (1 mM) buffer solution and transferred into an NMR tube containing 70 µL of a 1 mM trimethylsilylpropanoic acid (TSP) standard solution in deuterated water. Apolar fractions were dissolved in 700 µL of deuterated chloroform (CDCl₃) and transferred into NMR tubes. Each polar extract was resuspended in 630 µL PBS (1 mM) and transferred into an NMR tube containing 70 µL of a 1 mM TSP standard solution in deuterated water. Apolar fractions were dissolved in 700 µL CDCl₃ and transferred into NMR tubes.

**NMR Acquisition**

1D and 2D spectra were acquired using 400 MHz Prodigy (Bruker BioSpin GmbH, Rheinstetten, Germany). TOCSY and HMBC 2D experiments were acquired using a 400 MHz Bruker NMR spectrometer. One–dimensional (1D) proton spectra were acquired using the excitation sculpting sequence (Hwang and Shaka, 1995). In detail, a double–pulsed field gradient echo with a soft square pulse of 4 ms at the water resonance frequency was used. The gradient pulse had a duration of 1 ms and a total of 516 transients were added. In total 16,384 points were acquired with a spectral width of 8417.5 Hz. Time-domain data were all zero-filled to 32,768 points, and prior to Fourier transformation, an exponential multiplication of 0.8 Hz was applied.

For the two–dimensional (2D) TOCSY (Bax and Davis, 1985; Griesinger et al., 1988), a standard pulse sequence was used with a spinlock period of 64 ms, achieved with the MLEV–17 pulse sequence. For water suppression, the excitation sculpting sequence was used. In general, 256 equally spaced evolution-time period t₁ values were acquired, averaging 64 transients and resulting in a total of 2,048 measured points with 8403.36 Hz of spectral width. Time-domain data matrices were all zero-filled to 4,096 points in both dimensions, thus yielding a digital resolution of 2.04 Hz/pt. Before Fourier transformation, a Lorentz–to–Gauss window with different parameters was applied for all the experiments' t1 and t2 dimensions.

Natural abundance 2D HSQC (¹H–¹³C) spectra were recorded at 150.90 MHz for ¹³C, using an echo–anti echo phase-sensitive pulse sequence with adiabatic pulses for decoupling (Kay et al., 1992; Palmer et al., 1991) and presaturation for water suppression (Schleucher et al., 1994). 256 equally spaced evolution-time period t₁ values were acquired, averaging 96 transients of 2,048 points and using GARP4 for decoupling. The final data matrix was zero-filled to 4096 in both dimensions and apodized before Fourier transformation by a shifted cosine window function in t2 and t1 (to smooth the discontinuities at the beginning and end of the sampled time record). The linear prediction was also applied to extend the data to twice its length in t1. HSQC spectra in water were referred to the α–glucose doublet resonating at 5.24 ppm for ¹H and 93.10 ppm for ¹³C. For all spectra acquired in water, deuterated TSP (0.1 mM) was used as internal reference, which is assumed to resonate at δ
Metabolite Identification and Identification of Metabolite Shifts under Ocean Acidification Using Multivariate Analyses

Before performing multivariate statistical modelling, we automatically reduced the phased- and baseline-corrected $^1$H NMR spectra data down to 450 integral segments of 0.02 ppm each between the 0.50-9.50 ppm spectral region, excluding the water resonance (4.42-5.12 ppm) using the AMIX 3.9.15 software package (Bruker Biospin GmbH, Rheinstetten, Germany). After reducing NMR data, bins were normalized to the total spectrum area. The obtained data format, expressed by a matrix (X matrix), was then imported into SIMCA-P+14 package (Umetrics, Umeå, Sweden) where Principal Components Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were performed, after pareto scaling. We first applied unsupervised PCA to explore data trend and eventually exclude outliers (data not shown). Once homogeneity was assessed, we applied supervised OPLS-DA to emphasize categories discrimination, where dummy variables were assigned to define class belonging (Y matrix) that is, in this case, to investigate the metabolic differences in sponge species collected in diverse pH environmental conditions. Therefore, two regression models were created for each experimental context, one for C. reniformis and another for S. cunctatrix.

To delve into specifics, OPLS-DA is a potent technique for examining qualitative data structures. This method is especially adept at processing Omics data structures, including NMR data sets, for the purpose of discriminant analysis (Jonsson et al., 2005; Pérez-Enciso and Tenenhaus, 2003). One significant benefit of utilizing OPLS-DA is the simplicity with which the generated models can be interpreted. These models emphasize discriminant information that is condensed within their predictive components, facilitating easy comprehension (Boccard and Rutledge, 2013). The OPLS algorithm (Trygg and Wold, 2002) allows to integrate an orthogonal signal correction filter (Wold et al., 1998) to distinguish useful variations in the data set from noise, by separating predictive from non-predictive (orthogonal) variation (Bylesjö et al., 2006). Unexpected variations in NMR descriptor variables that are not correlated to a class response (here: control site versus vent site) are removed and variability related to experimental bias or biological variations are highlighted (Williams et al., 1987). In mathematical terms this is equivalent to removing systematic variation in X that is orthogonal to Y, where a quantitative relationship is investigated between a descriptor matrix X and a response matrix Y (Trygg and Wold, 2002). Each elaborated statistical model was computed with predictive and orthogonal components using five representative replicates per species and site. While the predictive component aimed at class
discrimination (mainly accounting for inter-class separation), the orthogonal component was responsible to estimate intra-class data dispersion. The resulting score plots revealed class separation for each sponge species between sites. Hierarchical S-plots were used to identify significant NMR variables from the X-matrix based on the OPLS-DA model to distinguish between two groups. The advantage of the S-Plot is that it describes the fitted covariance vector on the x-axis and the correlation coefficient vector on the y-axis, describing the contribution and correlation of the main NMR variables to group separation, respectively (Yang et al., 2013).

Based on the identified NMR variables, which were most discriminant for class separation, quantitative responses were predicted in relation to the defined categorical classes (Trygg and Wold, 2002). This revealed putative NMR variables, i.e. metabolites, which were likely up- or down-regulated in C. reniformis and S. cunctatrix in response to OA. The model qualities were evaluated by the goodness-of-fit parameter ($R^2$) and the goodness-of-prediction parameter ($Q^2$). For them, acceptable values must be $\geq 0.5$, with $|R^2-Q^2|< 0.2$ to 0.3 (Eriksson et al., 2001).

The final metabolites assignment to NMR variables was performed according to descriptive $^1$H NMR peaks by comparison of chemical signal shifts with literature data (Fan 1996) and/or an online database (Wishart et al. 2022). The chemical shifts of single metabolites of the $^1$H NMR spectrum were further validated with the 2D experiments, $^1$H-$^1$3C HSQC experiments (and the $^1$H-$^1$H TOCSY; not shown).

**Statistical analyses of relative metabolite shifts in response to OA**

To validate and assess the performance of the OPLS-DA predicted metabolite trends, a univariate t-test (with false-discovery rate corrected $p < 0.05$) was applied on normalized NMR bin intensities of each variable, representing the relative quantity of a metabolite. The intensities are determined as a sum of points included in a rectangular area whose base is determined by the bin size (here 0.02 ppm). Statistical analyses included 10 replicates per species and site. One replicate of S. cunctatrix sampled at the vent site yielded not enough biomass for a consistent chemical extraction and was excluded from downstream analyses. Statistical tests were performed in R studio v. 4.2.0. Visualization of relative abundance shifts of assigned metabolites were based on mean, normalized bin intensities per NMR variable. Plots were produced with ggplot2 (Wickham, 2016).
RESULTS

NMR SPECTRA OF C. RENIFORMIS AND S. CUNCTATRIX EXTRACTS

Representative $^1$H NMR profiles of C. reniformis are shown in Figure 3.6 and Figure 3.7, with the corresponding 2D $^1$H-13C interactions from the HSQC experiment in Figure 3.8. $^1$H NMR profiles of S. cunctatrix are shown in Figure 3.9 and Figure 3.10 and the $^1$H-13C HSQC experiment in Figure 3.11. NMR spectra were characterized by numerous sharp peaks, showing the complexity of sponge polar extracts. Since the NMR spectra showed qualitatively very similar signals across extracts from control and vent sites, only one exemplary spectrum per species is presented.

Mostly primary metabolites have been identified. A list of all assigned metabolites and according characteristic $^1$H and 13C resonances is provided in Table 3.1. NMR signals from most primary metabolites have been assigned to peaks in the chemical shift region between 0.9 and 4.5 ppm (Figure 3.7 and Figure 3.10). Identified signals for $\alpha$-amino acids included glycine (3.59 ppm), alanine (1.49 and 3.82 ppm), glutamate (2.35, 2.18 and 3.80 ppm), glutamine (2.18 and 2.47 ppm), lysine (1.71 ppm), threonine (1.34 and 4.30 ppm), tyrosine (0.90 ppm), aspartate (2.79 ppm), the non-proteinogenic $\alpha$-amino acid creatine (3.04 and 3.93 ppm), as well as the branched-chain amino acids valine (0.99 and 1.05 ppm), leucine (0.96, 1.73 and 3.77 ppm) and isoleucine (0.93 and 1.49 ppm). The amino acid derivative sarcosine, also known as N-methyl glycine was identified at 3.63 ppm. Further ubiquitously common primary metabolites of general central metabolism pathways were present in sponge extracts, including acetate (1.93 ppm), succinate (2.41 ppm), methylamine (2.63 ppm) and trimethylamine-N-oxide (3.27 ppm). In both sponge species, distinct signals were also present from creatine (3.04 and 3.93 ppm), choline (3.19 ppm) as well as characteristic triplet signatures from taurine (3.45 ppm).

Metabolites identified exclusively in C. reniformis included distinct peaks from histamine (3.08, 3.34, 7.24 and 8.19 ppm), together with Imidazole (7.28 and 8.21 ppm). In polar extracts from S. cunctatrix, strong signals were present for trigonelline (8.09, 8.84 and 9.12 ppm) along with Allantoin (6.01 and 5.39 ppm) at higher chemical shifts of the spectrum. Extraordinarily strong signals were recorded for betaine (3.27, 3.91 ppm). Lipids and lipoproteins heavily overlapped at 0.85 - 1.05 ppm.
Figure 3.6: $^1$H-NMR spectrum of *C. reniformis* polar extract in H$_2$O+D$_2$O. Enlarged view is provided for ppm shifts between 6.9 and 8.8 ppm for better resolution.
Figure 3.7: Metabolite assignment on $^1$H-NMR spectrum of *C. reniformis* polar extract in H2O+D2O. Shown is the part of the spectrum between 1 – 4 ppm, where most metabolites have been assigned. Abbreviation: DMG: Dimethylglycine
Figure 3.8: 2D HSQC spectrum of *C. reniformis* polar extract in H2O+D2O showing carbon-hydrogen interactions.
Figure 3.9: NMR spectrum of \textit{S. cunctatrix} polar extract in H2O+D2O. Shown are two representative spectra from acidified and control conditions.
Figure 3.10: Metabolite assignment on NMR spectrum of *S. cunctatrix* polar extract in H2O+D2O. Shown is the part of the spectrum between 1 – 4 ppm, where most metabolites have been assigned. Abbreviations: TMAO = Trimethylamine-N-oxide, GPC = Glycerophosphocholine
Figure 3.11: 2D HSQC spectrum of *S. cunctatrix* polar extract in H2O+D2O showing carbon-hydrogen interactions.
Table 3.1: $^1$H and $^{13}$C chemical shift assignment (δ, ppm) of metabolites found in $^1$H NMR and $^1$H-13C-HSQC-NMR spectra of *C. reniformis* and *S. cunctatrix* polar extracts. (i) Multiplicity of $^1$H NMR signal is indicated with letters s = singlet, d = triplet, tt = doublet, t = triplet, dt = doublet of triplets, q= quartet, m = multiplet, c = complex (ii) Numeration of H-Atoms according to the Human Metabolome Database (HMDB) (Wishart et al., 2022).

| Nr | Met. | $\delta$ $^1$H [1] | $\delta$ $^{13}$C | Group | Nr | Met. | $\delta$ $^1$H | $\delta$ $^{13}$C | Group |
|----|------|------------------|------------------|-------|----|-----------------|-----------------|-------|
| 1  | Glycine | 3.59 s | 42.4 | $\alpha$CH | 18  | Glc$^a$/PCr$^a$ | 3.04 s | 39.3 | NCH$_3$ |
|    |       |       |       |       | 19  | Cho$^a$/ | 3.60 | 66.8 | $\alpha$CH |
|    |       |       |       |       | PC$^a$ | 4.17 | 58.4 | $\beta$CH |
| 2  | Leucine | 3.77 t | 61.8 | $\alpha$CH | 20  | GPC$^a$ | 4.33 m | 66.3 | $\alpha$CH$_2$(P) |
|    |       | 1.73 m | 40.5 | $\beta$CH$_2$ |       |       | 3.68 dd |  -  | $\gamma$CH |
|    |       | 1.69 m | 24.8 | $\gamma$CH | 3.93 s | 54.4 | NCH$_3$ |
|    |       | 0.95 d | 21.6 | $\delta$CH$_3$ |       |       | 3.22 s | 54.4 | NCH$_3$ |
|    |       | 0.96 d | 22.2 | $\delta$CH$_3$ |       |       |       |       |       |
| 3  | Valine | 0.99 d | 17.3 | $\gamma$CH$_3$ | 21  | Glycerol | 5.47 | 95.1 | C$_6$H |
|    |       | 1.05 d | 18.44 | $\gamma$CH$_3$ |       |       |       |       |       |
| 4  | Threonine | 1.34 d | 20.45 | $\gamma$CH$_3$ | 22  | Taurine | 3.26 t | 38.1 | SCH$_2$ |
|    |       | 4.30 m | 65.67 | $\beta$CH | 3.45 t | 50.2 | NCH$_3$ |
|    |       | 3.59 d | 61.46 | $\alpha$CH |       |       |       |       |       |
| 5  | Lactate | 1.34 d | 20.2 | $\beta$CH$_3$ | 23  | Imidazole | 8.21 s | 147.8 | H$_9$[2] |
|    |       | 4.19 q | 69.1 | $\alpha$CH | 7.28 s | 123.9 | H$_6$, H$_7$[2] |
|    |       |       |       |       | 8.19 s | 136.5 |       |       |       |
| 6  | Lysine | 3.79 t | 54.7 | $\alpha$CH | 24  | Trehalose | 5.20 d | 94.2 | C$_1$H |
|    |       | 1.90 m | 30.2 | $\beta$CH$_2$ |       |       |       |       |       |
|    |       | 1.46 m | 21.9 | $\gamma$CH$_2$ |       |       |       |       |       |
|    |       | 1.71 m | 24.8 | $\delta$CH$_2$ |       |       |       |       |       |
|    |       | 3.02 t | 39.5 | $\omega$CH$_2$ |       |       |       |       |       |
| 7  | Alanine | 3.82 q | 51.33 | $\alpha$CH | 25  | Histamine | 3.08 m | 24.0 | H$_{13}$, H$_{14}$[2] |
|    |       | 1.49 d | 16.74 | $\beta$CH$_3$ | 3.34 t | 39.6 | H$_{11}$, H$_{12}$[2] |
|    |       |       |       |       | 7.24 s | 118.1 |       |       |       |
|    |       |       |       |       | 8.19 s | 136.5 |       |       |       |
| 8  | Beta- | 3.18 t | - | $\alpha$CH | 26  | UDP$^a$ | 4.40 | 84.3 | CH ribose |
|    | Alanine |       |       | $\beta$CH$_3$ | 5.98 | 88.7, 102.7 | C$_4$H ribose |
|    |       | 2.55 t | 39.2 |       | 7.96 | 141.27 | C$_6$ ring |
| 9  | Acetate | 1.93 s | 23.5 | $\beta$CH$_3$ | 27  | Tyrosine | 0.90 | - | H$_{22}$-H$_{24}$[2] |
|    |       |       |       |       | 6.88 d | 117.0 | C$_{3c}$ H ring |
|    |       |       |       |       | 7.20 d | 130.0 | C$_{2c}$ H ring |
| 10 | Glutamate | 3.80 t | 55.1 | $\alpha$CH | 28  | Methyl | 2.63 s | - | CH$_3$ |
|    |       | 2.18 dt | 27.4 | $\beta$CH |       |       |       |       |       |
|    |       | 2.18 dt | 27.4 | $\beta$CH |       |       |       |       |       |
|    |       | 2.35 t | 33.6 | $\gamma$CH$_2$ |       |       |       |       |       |
| 11 | Glutamine | 3.77 t | 55.1 | $\alpha$CH | 29  | Allantoin | 6.01 s | - | - |
|    |       | 2.18 c | 27.3 | $\beta$CH$_2$ |       |       |       |       |       |
|    |       | 2.47 c | - | $\gamma$CH$_2$ |       |       |       |       |       |
| 12 | Iso-Leucine | 1.98 m | 36.6 | $\beta$CH |       |       |       |       |       |
|    |       | 1.00 d | 15.4 | $\gamma$CH$_3$ |       |       |       |       |       |
|    |       | 1.45 m | - | $\gamma$CH |       |       |       |       |       |
|    |       | 0.96 t | 11.8 | $\delta$CH$_3$ |       |       |       |       |       |
13 Betaine (Glycine betaine) 3.27 s 54.2 CH₃ 3.91 s 68.3 α CH₂ 3.27 s 3.91 s 54.2 CH₃ 3.91 s 68.3 α CH₂
14 TMAO 3.27 s 61.6 H6-H14 31 Succinate 2.41 s 34.3 α,β CH₂
15 Sarcosine 2.75 s - CH₃ 3.63 s - CH₂ 32 Aspartate 2.79 dd 52.7 β CH₃
16 Uracil 7.56 d 144.8 H9 5.82 d 101.8 H10 33 DMG 2.91 s - CH₃
17 1-MNA³ 9.27 s 146.4 H19 8.99 d - H14 8.91 d - H16 34 MeOH 3.37 s 49.8 CH₃

³Abbreviations: Cr: Creatine; PCr: Phosphocreatine; Cho: Choline; PC: Phosphocholine; GPC: Glycerophosphocholine; Glc1P: Glucose-1-Phosphate; UDP: Uridine diphosphate; 1-MNA: 1-Methyl-Nicotinamide, TMAO: Trimethylamine-N-oxide; DMG: Dimethylglycine

**METABOLIC DYNAMICS IN HMA AND LMA SPONGE HOLOBIONTS IN RESPONSE TO OCEAN ACIDIFICATION**

**C. reniformis - metabolite trends**

The OPLS-DA in Figure 3.12 described the variation of *C. reniformis* from acidified versus control conditions with an accuracy of 72% (R² = 0.72) and a predictive ability of 20% (Q² = 0.20). The score plot showed a good discrimination and sample projection for all *C. reniformis* samples, except for the replicate VC5. The first component t[1] accounted for the main differences between *C. reniformis* control group at t[1] negative coordinates, versus the ‘treated’ class under acidified conditions, placed at positive t[1]. The orthogonal component to[1] mainly expresses the control class inhomogeneity. The loading plot in Figure 3.12B shows all NMR variables (metabolites) responsible for sample projection and clusters (Figure 3.12A) in the model. Most relevant variables were selected based on a good correlation between the principal component coordinates, i.e. the position of the sample spot projected in the plot, and the original NMR variables. The shown separation of NMR variables was predicted by the statistical model, but are based on real NMR signal variation.

To highlight a subset of metabolites, which were most responsible in characterizing the discrimination for control pH versus vent samples, NMR variables were selected using the correlation loading values of |p(corr)| > 0.7 from the OPLS-DA model classification (Figure 3.13).
Figure 3.12: OPLS-DA score plot of *C. reniformis* grown in control pH (8.04 – 8.05) and CO₂ vent pH (7.6 – 7.88) conditions. R² = 0.72 and Q²=0.20. A) The horizontal component t[1] of the PCA score plot captures the predicted variation between the groups, i.e. sponge individuals from vent versus control sites. The orthogonal component to[1] shows the non-predictive variation of NMR variables, thus displaying the within group variation. B) NMR variables responsible for sample projection and clustering.

Figure 3.13: S-plot of the OPLS-DA model of NMR variables vent vs. control in *C. reniformis*. The x-axis represents the contribution p[1], while the y-axis the correlation p(corr)[1] of the main NMR variables to group separation. Twelve down-regulated NMR variables were selected from this S-plot.

Most relevant compounds from *C. reniformis* polar profiles involved in the biochemical response to the acidified environment are listed in Table 3.2. Interestingly, an overall downregulation of common primary metabolites, mainly amino acids were found including, arginine, valine, glycine, threonine, the beta amino acid β-alanine, the simplest primary amine methylamine and the histidine derived histamine. The organic compound creatine and its phosphorylated form phosphocreatine showed analogously downregulation at the vent site. The ubiquitous anion acetate was found to decrease under OA, along with the organic compound taurine.
Table 3.2: Selected NMR variables down-regulated in acidified vs control conditions from the OPLS-DA model with corresponding metabolite assignment from *C. reniformis* polar profiles involved in the biochemical response to OA.

<table>
<thead>
<tr>
<th>Downregulated metabolites (chemical shift in ppm)</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate (1.95)</td>
<td><img src="image" alt="Acetate Chemical Structure" /></td>
</tr>
<tr>
<td>Arginine (1.93)</td>
<td><img src="image" alt="Arginine Chemical Structure" /></td>
</tr>
<tr>
<td>Aspartate (2.85)</td>
<td><img src="image" alt="Aspartate Chemical Structure" /></td>
</tr>
<tr>
<td>Taurine (3.33 - 3.31)</td>
<td><img src="image" alt="Taurine Chemical Structure" /></td>
</tr>
<tr>
<td>Histamine (3.09)</td>
<td><img src="image" alt="Histamine Chemical Structure" /></td>
</tr>
<tr>
<td>Valine (0.99; 1.03)</td>
<td><img src="image" alt="Valine Chemical Structure" /></td>
</tr>
<tr>
<td>β-alanine (2.55)</td>
<td><img src="image" alt="β-alanine Chemical Structure" /></td>
</tr>
<tr>
<td>Methylamine (2.63)</td>
<td><img src="image" alt="Methylamine Chemical Structure" /></td>
</tr>
<tr>
<td>Lactate / Threonine (1.33; 1.35)</td>
<td><img src="image" alt="Lactate Threonine Chemical Structure" /></td>
</tr>
<tr>
<td>Glycine (3.65 - 3.55)</td>
<td><img src="image" alt="Glycine Chemical Structure" /></td>
</tr>
<tr>
<td>Phosphocreatine (3.01)</td>
<td><img src="image" alt="Phosphocreatine Chemical Structure" /></td>
</tr>
<tr>
<td>Creatine (3.05; 3.93)</td>
<td><img src="image" alt="Creatine Chemical Structure" /></td>
</tr>
</tbody>
</table>

**S. CUNCTATRIX - METABOLITE TRENDS**

To pinpoint and to evaluate molecules responsible for the metabolic alterations induced in *S. cunctatrix* sponge inhabiting in the CO₂ vent site, OPLS-DA was performed on NMR spectra (Figure 3.14). The statistical model resulted in high predictive accuracy with the parameters $R^2 = 0.96$ and $Q^2 = 0.92$. The score plot in Figure 3.14A shows sample projection with a strong discrimination across both sites. The first component t[1] accounts for the main differences between *S. cunctatrix* control pH
class at $t[1]$ negative coordinates, versus the $CO_2$ vent class, placed at positive $t[1]$. The orthogonal component to $t[1]$ mainly expresses the $CO_2$ vent in homogeneity. Figure 3.14B shows the NMR variables responsible for sample projection and clustering in the model.

![Figure 3.14: Score scatter plot of principal components analyses (PCA) of S. cunctatrix grown in control pH (8.04 – 8.05) and $CO_2$ vent pH (7.6 – 7.88) conditions. $R^2 = 0.72$ and $Q^2 = 0.20$. A) The horizontal component $t[1]$ of the OPLS-DA score scatter plot captures the predicted variation between the groups, i.e. sponge individuals from vent versus control sites. The orthogonal component $t[1]$ shows the non-predictive variation of NMR variables and displays within group variation. B) NMR variables responsible for sample projection and clustering.](image)

Figure 3.15: S-plot of the OPLS-DA model of NMR variables vent vs. control in S. cunctatrix. The x-axis represents the contribution $p[1]$, while the y-axis the correlation $p(corr)[1]$ of the main NMR variables to group separation. Six down-regulated and 8 up-regulated NMR variables were selected from this S-plot.

To highlight the subset of most discriminant metabolites in S. cunctatrix, NMR variables were selected using a correlation loadings value $|p(corr)| > 0.7$ in the OPLS-DA model classification (Figure 3.15). The most relevant compounds involved in the biochemical response to the acidified environment are listed in Table 3.3.

The OPLS-DA model predicted various amino acids to be upregulated in response to OA, including alanine, arginine and the branched-chain amino acids valine, leucine and isoleucine, as well as the N-trimethylated amino acid betaine (N,N,N-Trimethylglycine). Lipids were found to be more abundant in sponges from the acidified environment. Instead, predicted down-regulated metabolites included taurine, creatine, trigonelline, allantoin, AMP and GPC.
Table 3.3: Selected up- and downregulated NMR variables in acidified vs control conditions from the OPLS-DA model with corresponding metabolite assignment from *S. cunctatrix* polar profiles involved in the biochemical response to OA. Abbreviations: AMP= Adenosine monophosphate; GPC= Glycerylphosphorylcholine; BCAA= branched-chain amino acids (valine, leucine and isoleucine). *FDR corrected T-Tests confirmed significance of relative metabolite shift.

<table>
<thead>
<tr>
<th>Downregulated metabolites (chemical shift in ppm)</th>
<th>Chemical Structure</th>
<th>Upregulated metabolites</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine* (3.33-3.31)</td>
<td><img src="image1" alt="Taurine Structure" /></td>
<td>Lipids* (0.89)</td>
<td><img src="image2" alt="Lipids Structure" /></td>
</tr>
<tr>
<td>Creatine* (3.05; 3.93)</td>
<td><img src="image3" alt="Creatine Structure" /></td>
<td>BCAA’s: Valine* (0.99; 1.03)</td>
<td><img src="image4" alt="Valine Structure" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leucine * (0.97)</td>
<td><img src="image5" alt="Leucine Structure" /></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isoleucine* (0.93)</td>
<td><img src="image6" alt="Isoleucine Structure" /></td>
</tr>
<tr>
<td>Trigonelline* (8.87)</td>
<td><img src="image7" alt="Trigonelline Structure" /></td>
<td>Alanine (1.49)</td>
<td><img src="image8" alt="Alanine Structure" /></td>
</tr>
<tr>
<td>Allantoin* (6.03)</td>
<td><img src="image9" alt="Allantoin Structure" /></td>
<td>Betaine* (glycine betaine; 3.91)</td>
<td><img src="image10" alt="Betaine Structure" /></td>
</tr>
<tr>
<td>AMP* (8.61)</td>
<td><img src="image11" alt="AMP Structure" /></td>
<td>Lactate/Threonine* (1.33; 1.35)</td>
<td><img src="image12" alt="Lactate/Threonine Structure" /></td>
</tr>
<tr>
<td>GPC (3.23)</td>
<td><img src="image13" alt="GPC Structure" /></td>
<td>Arginine* (1.93)</td>
<td><img src="image14" alt="Arginine Structure" /></td>
</tr>
</tbody>
</table>
**RELATIVE ABUNDANCES OF METABOLITE SHIFTS**

The relative abundance shifts of OPLS-DA suggested metabolites in *C. reniformis* and *S. cunctatrix* are displayed in Figure 3.16 and Figure 3.17, respectively. Statistical validation of semi-quantitative shifts in response to OA revealed no significant changes for *C. reniformis*. Instead for *S. cunctatrix*, most predicted metabolites showed statistically different relative abundances in CO$_2$ vent versus control pH.

![Relative metabolite abundance in *C. reniformis*](image1)

**Figure 3.16:** Relative metabolite abundance in *C. reniformis* ($n = 20$) across control and vent site. No significant metabolite shifts were detected. Boxplots show range (whiskers), median (bold line), and interquartile range (box height).

![Relative metabolite abundance in *S. cunctatrix*](image2)

**Figure 3.17:** Relative metabolite abundance in *S. cunctatrix* ($n = 19$) across control and vent site. Data is presented on a log scaled x axis. Boxplots show range (whiskers), median (bold line), and interquartile range (box height). *Significance according to t-test (FDR corrected $p < 0.05$).
**DISCUSSION**

The first insights into the chemical compositions of the sponge species *C. reniformis* and *S. cunctatrix* showed their metabolic complexities and allowed to identify a subset of metabolites according to unique chemical shifts. Numerous primary metabolites were identified in both species, including sugars, amino acids and their derivatives, as well as common organic acids such as acetate and lactate.

**IDENTIFIED METABOLITES IN C. RENIFORMIS**

In the metabolome of *C. reniformis*, numerous common primary metabolites were present in similar relative abundance across vent and control site. The organic osmolytes dimethylglycine (DMG) and taurine were abundant across both sites in similar abundances. Metabolites belonging to common central metabolism, such as glutamate, pyroglutamate, succinate were present, as well as the RNA nucleic acid uracil and the phospholipid compound choline. Identified amino acids included threonine, tyrosine, alanine and beta-alanine, lysine, glycine, as well as all branched-chain amino acids (isoleucine, valine, leucine) and the amino acid derivative creatine. Amino acids are widely transported into marine invertebrates across their body surfaces (Preston, 1993) and their exogenous uptake was shown to be reduced in the seep-sensitive sponges species *Styloissa flabelliformis* when inhabiting natural CO$_2$ vent sites (Botté et al., 2019). Metabolic responses of this species further suggested a reduced degradation of host-derived creatine, creatinine and taurine. In contrast, *C. reniformis* showed similar abundances of these compounds across sites, indicating that OA did not negatively impact amino acid metabolism. The diverse amino acid pool in *C. reniformis* may allow sponge-associated symbionts to metabolize amino acids into diverse bioactive secondary metabolites (e.g. Hedner et al., 2006; Phyo et al., 2018; Ueberlein et al., 2014). This might finally supply the holobiont with ecological advantages, e.g. for chemical defense based on deterrent, cytotoxic and/or antibiotically active compounds (Paul et al., 2011; Proksch et al., 2010; Thoms et al., 2006, 2004).

Metabolites which have been exclusively present in *C. reniformis*, but not in *S. cunctatrix*, included the histidine-derived histamine and the alkaloid imidazole. Histamine is a neurotransmitter widely distributed in mammals, plants and bacteria. Histamine derivatives have been found in the tropical sponge *Aplysina sp.* (Santalova et al., 2010). As an important signal molecule, histamine plays a key role in inter-domain and inter-species communication (Reviewed in Krell et al., 2021). Many bacteria are able to synthesize and secrete histamine, which in turn can modulate their host’s immune responses (Barcik et al., 2019; Ferstl et al., 2014). Recent studies showed that bacteria are able to sense histamine, which triggers chemoattraction (Corral-Lugo et al., 2018) and the
regulation of virulence-gene expression (Y. Wang et al., 2021), finally regulating multiple metabolic and physiological processes in bacteria and their hosts (McFall-Ngai et al., 2013; Rosier et al., 2016). The heterocyclic imidazole ring was further identified only in C. reniformis extracts. Imidazole containing alkaloids have already been isolated and characterized in other sponge species, Leucetta sp. (Akee et al., 1990; Edrada et al., 2003; Gross et al., 2002; Hassan et al., 2004; Tsukamoto et al., 2007), Clathrina (Roué et al., 2010) and Agelas oroides (Fattorusso and Taglialetela-Scafati, 2000). Imidazole rings are present in a plethora of alkaloids, such as preclathridines, clathridines, naamidines, isonaamidines, naamines, isonaamines, and pyronaamidine (Alvi et al., 1993; Carroll et al., 1993; Ciminiello et al., 1989; He et al., 1992; Roué et al., 2010). Different biological activities have been assigned to 2-aminoimidazole-based alkaloids, including cytotoxicity (Tsukamoto et al., 2007), antifungal (Ciminiello et al., 1989), anti-inflammatory (Chan et al., 1993), and antitumor activities (Copp et al., 1998). There is ongoing interest of different research groups in synthesizing these marine alkaloids (Bjørsvik and Sandtorv, 2014; Lovely and Harmata, 2007) and it would be worth searching in future studies, if C. reniformis solely produces imidazole, or also derived alkaloids with possible ecological relevance as a secondary metabolite and possible biomedical application.

Comparing the NMR spectra of C. reniformis sampled across control and CO₂ vent sites, revealed no significant shifts in relative metabolite abundances. Although the multivariate data analyses using OPLF-DA predicted an overall downregulation of common primary metabolites, statistical testing could not validate these results. According to the HMA dichotomy, C. reniformis profits from a highly diverse microbiome, which likely enhances the probability of functional convergence for central metabolic pathways (Fan et al., 2012). Differences in microbial gene abundances have been reported in HMA and LMA sponges (Bayer et al., 2014). Changes in environmental conditions may be less likely to significantly affect key metabolic processes in HMA sponges, facilitating them to maintain molecular homeostasis.

**METABOLIC SHIFTS IN S. CUNCTATRIX IN RESPONSE TO OCEAN ACIDIFICATION**

The metabolome of the LMA sponge S. cunctatrix showed significant changes of different key metabolite levels across CO₂ vent and control pH sites. Lower metabolite abundances in sponges inhabiting the vent site included the amino acid derivatives creatine, the alkaloid trigonelline, the organic osmolytes taurine and allantoin, as well as central metabolites such as adenosine monophosphate (AMP) and glycerylphosphorylcholine (GPC). Increased levels have been revealed for mainly primary metabolites, such as amino acids, the organic osmolyte glycine betaine, as well as choline and the CH₂/CH₃ groups representing lipids. In the following, the main metabolites identified in S. cunctatrix and their biological relevance, as well as association to vent or control pH site will be described in detail. Revealed metabolites are summarized in Table 3.4
**Osmoregulatory Metabolites**

Taurine, an osmoregulating amino acid with lower concentrations in *S. cunctatrix* inheriting the vent site in comparison to the control pH site. Taurine has been found in a broad suite of sponge species and represents one of the most abundant free amino acids in sponges (Hooper et al., 1992), comprising up to 74% of the free amino acid pool. This metabolite is suggested to be key in host-symbiont interactions between the sponge and its associated microbiome. Sponge symbionts metabolize taurine to acquire sulfite (Dharamshi et al., 2022; Moeller et al., 2022). Recent evidence was found in the *lanthella basta* holobiont, where ammonia was derived from taurine and exported by gammaproteobacterial symbionts for immediate oxidation by dominant ammonia-oxidizing thaumarchaeal symbionts (Moeller et al., 2022). Taurine can be either produced by the sponge host itself, or be imported from the seawater into the sponge holobiont (Botté et al., 2019). In agreement with our results, the sponge *Stylissa flabelliformis* from the CO$_2$ vents in Papua New Guinea (Upa-Upasina), revealed that OA induced reductions in host taurine production and a reduced potential for microbial taurine dissimilation (Botté et al., 2019). The sponge microbiome showed a significant decrease in gene abundances encoding key enzymes for the taurine degradation pathway (Ruff et al., 2003), such as the taurine-pyruvate aminotransferase. This enzyme is responsible for the taurine degradation into sulfoacetaldehyde and L-alanine. Further metabolization into sulfite and acetate phosphate is subsequently performed by the sulfoacetaldehyde acetyltransferase. Decreased levels in the microbial gene encoding cysI, further lead to reduced conversion of sulfite into sulfide (Botté et al., 2019). This overall decreases the potential for the sponge holobiont to use taurine and produce sulfide, and authors suggested that could contribute to the poor performance of *S. flabelliformis* at the CO$_2$ vent site (Botté et al., 2019). These findings from Botté et al. (2019) seem to match our observations of *S. cunctatrix* in the vent site.

Aquarium experiments with oysters exposed to elevated pCO$_2$ revealed divergent patterns of elevated osmolyte levels, including increased taurine and homarine, along with depleted hypotaurine levels. In the study authors suggested that severe osmotic stress caused by elevated pCO$_2$ exposure, could be counteracted by increasing osmolytic metabolites (Wei et al., 2015). Taurine is considered as an important osmoregulating metabolite, providing cell protection (Ripps and Shen, 2012) and maintaining constant cell volumes under changing environmental osmolality (Pierce, 1982; Ripps and Shen, 2012) in marine invertebrates, including bivalve species (Gilles, 1972) and sponges (Shinagawa et al., 1992). However, the two weeks OA experiment by Wei et al. (2015) showed only significant results at extreme CO$_2$ enrichment at 2008 ppm CO$_2$ (pH 7.6), but not at pCO$_2$ 1036 ppm (pH 7.8). This experimental setting contradicts good-practice recommendations, where a use of realistic pCO$_2$ values are suggested, ranging between 450 and 1000 ppm (~pH 7.8) for acidification experiments (McElhany and Shallin Busch, 2013; Riebesell et al., 2011). It is possible
that at particularly high CO$_2$ levels, taurine expression was upregulated in oyster hepatopancreatic cells as a stress response for direct cell osmoregulation. Instead, sponges inhabiting natural CO$_2$ vents with milder OA conditions may have showed a taurine decrease due to chronic indirect effects, such as changes in the microbiome and/or ammonia and sulfide metabolism.

Similar osmoregulatory functions are attributed to the cyclic betaine trigonelline, which was also decreased in $S$. cunctatrix at the vent site. Trigonelline is usually common among higher plants (Mazzafera, 1991; Zhou et al., 2012) and was found in marine kelps (Blunden et al., 2012). This alkaloid is also common among different marine organisms, including crabs (Draper and Weissburg, 2022), mollusks (Suwetja et al., 1989), hydroids (Berking, 1987) and sponges (Cafieri et al., 1998; Mohanty et al., 2020). In the intertidal sponge *Halichondria japonica*, trigonelline seems to play a significant role in intracellular osmoregulation (Shinagawa et al., 1992). According to Whiteley (2011), marine species that are weak ionic and osmoregulators have limited capabilities to compensate for acid–base changes and become more susceptible to negative effects of OA. As a consequence, the reduced concentrations of taurine and trigonelline in *S. cunctatrix* are likely to require compensation by other osmoregulatory compounds to maintain intracellular homeostasis under OA.

Betaines are likely to take over this function, and their increasing concentrations may contribute to osmoregulation in *S. cunctatrix* at the vent sites. Many naturally occurring betaines serve as organic osmolytes, and are either synthesized or taken up from the environment by cells to protect themselves against osmotic stress, drought, high salinity, or high temperature (Wishart et al., 2022). Elevated betaine concentrations were also reported in the sponges *Halichondria okadai* and *H. japonica*, after exposure to hyperosmotic stress (Shinagawa et al., 1992), while the pools of free amino acid and betaines decreased in hypoosmotic conditions. Along with these findings, an increase in betaine was recently observed in crustacean larvae, which have been grown under increased pCO$_2$ levels in controlled aquarium conditions. This work further proposed that osmotic and acid-base challenges were compensated via the upregulation of intracellular organic osmolytes (Noisette et al., 2021).
Table 3.4: Metabolite shifts in *S. cuneatrich* in response to ocean acidification.

<table>
<thead>
<tr>
<th>Downregulated metabolites ⧫</th>
<th>Metabolic Pathway</th>
<th>Possible consequences for <em>S. cuneatrich</em> at CO₂ vent</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine</td>
<td>Most abundant free amino acids in sponges</td>
<td>Reduced amino acid pool</td>
<td>(Hooper et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Sulfite source for sponge symbionts</td>
<td>Reduced sulfite availability: Negative impact an amino acid biosynthesis and reduced growth and body size</td>
<td>(Dharamshi et al., 2022; Moeller et al., 2022)</td>
</tr>
<tr>
<td></td>
<td>Ammonia source for sponge symbionts</td>
<td>Reduced ammonia availability: Negative impact an amino acid biosynthesis and reduced growth and body size</td>
<td>(Moeller et al., 2022)</td>
</tr>
<tr>
<td>Osmoregulation</td>
<td></td>
<td>Osmotic stress</td>
<td>(Ripps and Shen, 2012)</td>
</tr>
<tr>
<td>Trigonelline</td>
<td>Osmoregulation</td>
<td>Osmotic stress</td>
<td>(Shinagawa et al., 1992; Wishart et al., 2022)</td>
</tr>
<tr>
<td>Creatine</td>
<td>Key nitrogenous compound for sponge symbionts</td>
<td>Reduced nitrogen metabolism in symbionts: Negative impact an amino acid biosynthesis and reduced growth and body size</td>
<td>(Botté et al., 2019)</td>
</tr>
<tr>
<td></td>
<td>ATP recycling in spermatoocytes and choanocytes</td>
<td>Decreased ATP levels, energy homeostasis; reduced flagellar motility in spermatoocytes and choanocytes → speculations: negative impact on reproduction and filter-feeding activity</td>
<td>(Wishart et al., 2022)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Salomons and Wyss, 2007)</td>
</tr>
<tr>
<td>Allantoin</td>
<td>Marker of oxidative stress</td>
<td>lower oxidative stress response</td>
<td>(Wishart et al., 2022)</td>
</tr>
<tr>
<td></td>
<td>Stimulation of tissue growth (wound healing)</td>
<td>Less stimulation of healthy tissue growth</td>
<td>(Wishart et al., 2022)</td>
</tr>
<tr>
<td>AMP</td>
<td>Energy metabolism (intracellular): Activation of catabolic metabolism pathways via AMPK</td>
<td>Increased metabolic rate</td>
<td>(Hardie et al., 2006)</td>
</tr>
<tr>
<td>Upregulated metabolites</td>
<td>Metabolic Pathway</td>
<td>Possible consequences for <em>S. cunctatrix</em> at CO$_2$ vent</td>
<td>References</td>
</tr>
<tr>
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<tr>
<td>Betaine</td>
<td>Osmoregulation</td>
<td>Osmotic stress protection</td>
<td>(Wishart et al., 2022)</td>
</tr>
<tr>
<td>BCAAs: Valine, Leucine, Isoleucine</td>
<td>Protein synthesis and degradation</td>
<td>Possibly indicators for increased protein degradation</td>
<td>(Wei et al., 2015)</td>
</tr>
<tr>
<td>Arginine</td>
<td>Metabolized to urea and ammonium</td>
<td>Increased ammonium oxidation by associated symbionts, such as archaea</td>
<td>(Vajrala et al., 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Support biosynthesis pathways and the production of amino acids</td>
<td>(Botté et al., 2019).</td>
</tr>
<tr>
<td></td>
<td>Immediate precursor of nitric oxide</td>
<td>Mediation of larval settlement $\rightarrow$ reproduction, survival</td>
<td>(Song et al., 2021)</td>
</tr>
<tr>
<td></td>
<td>Produced during dephosphorylation of arginine phosphate</td>
<td>Increased intracellular energy pools</td>
<td>(Urish, 2013)</td>
</tr>
<tr>
<td>Lactate</td>
<td>Product of microbial anaerobic metabolism</td>
<td>Increased abundance or activity of lactate producers and anaerobic respiration</td>
<td>(MacFarlane and Gibson, 1991) (Kumari and Kozyrskyj, 2017)</td>
</tr>
<tr>
<td>Lipids</td>
<td>Energy storage, cell membrane component</td>
<td>Support sponge survival</td>
<td>(Bennett et al., 2018)</td>
</tr>
<tr>
<td>Choline</td>
<td>Cell membrane component: phosphatidylcholine</td>
<td>Lower membrane fluidity</td>
<td>(Hazel, 1995; Hofmann and Todgham, 2010; Kraffe et al., 2007)</td>
</tr>
</tbody>
</table>
**Amino Acids and Energy Metabolism**

Creatine levels decreased in *S. cunctatrix* at the vent site. This metabolite is produced by sponge hosts along with creatinine (Sona and Ellington, 2004), and represent a key nitrogenous compound for associated symbionts. It was shown that in CO₂ vents, the degradation of host-derived creatine and creatinine was reduced for the sponge *Styliissa flabelliformis*. This in turn means, that the sponge-associated microbiome is worse at using these nitrogenous compounds, suggesting a reduced benefit from sponge-derived creatine and creatinine fuelling nitrogen metabolism (Botté et al., 2019). The overall reduction in relative creatine levels in *S. cunctatrix* likely also reduced urea and ammonium production within the sponge holobiont, finally challenging biosynthesis pathways and growth (Botté et al., 2019). Increased urea is favourable for biomass production (Chen et al., 2020), whereas high ammonium levels were shown to fosters the growth of sponge microbiota, serving as a food source in *Vazella pourtalesii* (Maldonado et al., 2021). All in all, decreased creatine levels likely contributed to the reduced growth and body size of *S. cunctatrix* at the vent site.

**Figure 3.18**: Spatial ATP buffering by the CK/PCr system in spermatoocytes and choanocytes. Abbreviations: CK: creatine kinase; Cr: Creatine; PCr: Phosphocreatine; Mt: mitochondrion; N: nucleus; oxphos: oxidative phosphorylation. Picture from Salomons and Wyss (2007).

Creatine and its phosphorylated form, phosphocreatine play further important physiological roles in sponges, supporting ATP buffering to support energy homeostasis in spermatoocytes for external fertilization and choanocytes for water current generation (Hoffman and Ellington, 2005; Salomons and Wyss, 2007). Both cell types consume energy due to flagellar movements, and mediate the ATP buffering system via creatine kinase isoform systems to synthesize creatine. As visualized in Figure
3.18, phosphocreatine transfers the phosphate group to ADP and ATP is recycled and provides energy for flagellar motility. It should be investigated whether OA associated decreases in creatine levels could negatively impact the reproduction in the oviparous sponge *S. cunctatrix* (Lévi, 1976; Riesgo et al., 2014) or reduce water pumping rates and consequently filter-feeding efficiencies.

Amino acids such as branched-chain amino acids (BCAA) and arginine were increased at the vent site. Similar results were obtained for oysters exposed to high pCO2 treatments, where elevated amino acids were discussed as indicators for increased protein degradation as a stress response (Wei et al., 2015). Elevated amino acids in oysters were mainly aspartate, threonine, alanine and BCAAs.

In contrast, the vent adapted sponge *Stylissa flabelliformis* and its microbiome exhibited reduced potential for the uptake of exogenous amino acids. The response included a lower relative abundance of importers for arginine/ornithine, BCAAs and thiamine. These results imply that amino acid concentrations inside of the sponge holobiont tend to decrease. Since the sponge microbiomes of both examined vent species (*Stylissa flabelliformis* and *Coelocarteria singaporensis*) seemed to have a restricted capacity to use exogenous organic compounds for energy production and biosynthesis, their conclusions underlined that sponge hosts likely retained carbohydrates and amino acids derived from filter-feeding and de novo biosynthesis, rather than providing them to the symbionts (Botté et al., 2019). Their results further support, that the seep-tolerant sponge *Coelocarteria singaporensis* dominantly performed archaeal-driven arginine metabolism at the CO2 vent site via glutamate-supported arginine biosynthesis. This outcome seemed to agree with our findings, which revealed elevated arginine levels in *S. cunctatrix* at the CO2 vent. In future experiments, archaeal genes could be an interesting target to shed light on gene regulation involved in arginine metabolism and synthesis.

Increased availability of arginine within the holobiont increases urea and ammonium levels, which in turn fuel ammonium oxidation by associated symbionts, such as archaea (Vajrala et al., 2013) supporting biosynthesis pathways and the production of amino acids (Botté et al., 2019). These cascading effects could counteract the beforehand discussed decrease in biosynthesis pathways due to decreased taurine and sulfide availability.

Increased arginine levels might indicate an adaptive trait in *S. cunctatrix*, supporting reproduction and survival under OA stress. This assumption is based on a recent study, which showed a central role of arginine in the production of the signalling molecule nitric oxide, which is necessary for the settlement of sponge larvae and finally supports reproduction and the sponge life cycle. Bacterial symbionts synthesize arginine from citrulline and supply this metabolite back to their sponge hosts (Song et al., 2021).

Arginine levels, as well as AMP concentrations, are negatively correlated to the enzyme carbonic anhydrase and can further indicate a stress response under OA. This was shown in a study of the
marine polychaete *Sabella spallanzanii*, which was exposed to high pCO$_2$ conditions in vent systems off Ischia Island. This species increased its energy metabolism, accompanied by a significant decrease in carbonic anhydrase concentration, which is suggested to indicate that this polychaete may struggle with future high pCO$_2$ conditions (Turner et al., 2015). The results of this study are in accordance with our results, which showed increases in arginine levels, but contradict decreased AMP concentrations in *S. cunctatrix* inhabiting the vent site. Elevated arginine levels, likely evidence the contribution of high energy phosphagens to increased intracellular energy pools (Urich, 2013), whereby arginine is set free due to dephosphorylation of arginine phosphate, finally increasing relative arginine concentrations (Turner et al., 2015).

Responses of ATP and AMP levels in sponges under OA have not been investigated yet. However, in polychaete species, increased ATP levels in response to high pCO$_2$ conditions are discussed as a consequence of an increased metabolic rate under OA (Calosi et al., 2013). Same results were found in the Dungeness crab, which showed increased ATP production after low pH exposure (Trigg et al., 2019). In *S. cunctatrix*, decreased AMP levels coupled with increasing ATP levels suggest similar cellular responses. Future investigations of the energy metabolism and associated target enzymes in *S. cunctatrix* could be a useful to better understand intracellular mechanisms and their role in energy homeostasis in response to OA.

**Anaerobic metabolism**

Increased levels of lactate in *S. cunctatrix* indicate a stimulation of anaerobic metabolism within the holobiont. Lactate biosynthesis via the enzyme lactate dehydrogenase helps generating ATP, while disposing excess reducing power from NADH by converting it to NAD$^+$ (MacFarlane and Gibson, 1991). Microbes are able to synthesize lactate from different available substrates, such as lactaldehyde, acetate, sugars and pyruvate. Some bacteria produce lactate under abundant food availability (sugars), but formic acid under low substrate abundances. Lactate levels are additionally fine-tuned by the crosstalk between lactate producers, lactate utilizers and physiological conditions (Kumari and Kozyrskyj, 2017). As a consequence, increased lactate levels could indicate that microbial remodelling under OA shifted towards an increased abundance or activity of lactate producers within the sponge holobiont.

Increased lactate production under OA was also found in scallops after 45 days of exposure to acidification (pH 7.5). The increased lactate dehydrogenase activity represented a result of energy modulation in these molluscs by stimulating their anaerobic metabolism (Liao et al., 2019). In contrast, decreased concentrations of lactate and succinate, main products of anaerobiosis in annelids (Urich, 2013), were found in polychaetes exposed to OA at the vent sites off Ischia Island. These invertebrates had to significantly increase their aerobic capacity to sustain energy release (Turner et al., 2015).
**LIPID METABOLISM**

A higher relative abundance of lipids was predicted based on increasing signals from lipidic -CH₃ and -CH₂ groups. NMR signals assigned to choline were further upregulated in *S. cunctatrix* from the vent site and likely hint to an upregulation of phosphatidylcholine, the most abundant phospholipid in eukaryotic cell membranes (Aktas et al., 2010; Gibellini and Smith, 2010; Ridgway, 2013). Lipids and fatty acids are likely important components in sponge stress responses and may support sponge survival under future climate conditions (Bennett et al., 2018). Sponges with greater concentrations of storage lipids, phospholipids, sterols and elevated concentrations of long-chain polyunsaturated fatty acids, are more resistant to ocean warming, but little is known how the composition of lipids actually responds to OA. Cell membranes form a permeable barrier for cells and their organelles and the lipidic bilayer seems to be sensitive to environmental stressors (Hazel, 1995). The remodelling of membrane lipids, with changes in head group compositions can be part of stress responses, whereby increased choline concentrations lower the membrane fluidity (Hazel, 1995; Hofmann and Todgham, 2010; Kräffe et al., 2007). It can be speculated that a ‘stronger’ cell barrier could be involved in a protection mechanism by generally decreasing the exchange of compounds with the acidified environment.

Opposite results were obtained in a recent study, where bivalve larvae were exposed to elevated pCO₂ and showed a decrease in lipid abundances across different lipid classes. Physiological responses were accompanied by high mortality rates and suggested that increased energy demands are predicted to cause lower lipid levels response to pCO₂ (Gibbs et al., 2021). Stress responses to elevated temperatures on coral larvae instead showed that more energetic lipids accumulate at elevated temperatures despite still experiencing increased mortality. This work suggested that larvae are unable to use lipids under elevated pCO₂ (Graham et al., 2017).

The effects of elevated pCO₂ on the lipid composition in sponges is not well understood and needs further investigation. Since lipids are important structural components for cell membranes, energy reserves, nucleic acid transcription and other important cellular processes, the ‘lipidome’ is likely playing a vital role in the functioning of marine invertebrates (Laudicella et al., 2020). Applying LC-MS based metabolomics on apolar extracts from *S. cunctatrix* will allow to obtain a better picture of the composition of lipophilic compounds and their dynamical shifts in response to OA.

**OXIDATIVE STRESS MARKER**

Allantoin, a final product of purine metabolism, decreased at the vent site in *S. cunctatrix*. This metabolite commonly occurs in plants (Braca et al., 2003; Maksimović et al., 2004), in most mammals (Wishart et al., 2022), and in sponge species, such as *Cymbastella cantharella* (Mourabit et al., 1997), *Tethya aurantia* (Weber et al., 1981), *Axinellidae gen. sp.* (Reshetnyak et al., 1988). In
1838, allantoin was already mentioned as an oxidation product of uric acid and was first found as an endogenous metabolite in dogs, cats, rabbits, and monkeys (Uyeno, 1917) and small amounts in humans (Folin et al., 1924). It was demonstrated that under oxidative stress, allantoin modulates the activity of enzymes belonging to the antioxidant system and increases intracellular H$_2$O$_2$, a second messenger promoting growth factors, proliferation and tyrosine kinases (Dröge, 2002; Shestopalov et al., 2006). Simultaneously, allantoin counterbalances possible H$_2$O$_2$ induced mutagenesis and toxicity (Gus’kov et al., 2002). It was shown that endogenic allantoin synthesis increases in humans after high physical activity (Hellsten et al., 2001; Mikami et al., 2000), likely due to a higher concentration of free reactive oxygen species (Benzie et al., 1999; Lagendijk et al., 1995; Marklund et al., 2000). Since allantoin decreased in S. cunctatrix at the vent site, a lower oxidative stress response can be suggested. It is likely that other cellular mechanisms are required to modulate antioxidant systems. Generally, the relevance of oxidative stress in sponges under ocean acidification is poorly investigated. Long-term exposure of bivalves to moderately elevated CO$_2$ (800 ppm) and warming (+5°C) only showed minimal effects on their cellular redox status and levels of oxidative stress biomarkers (Matoo et al., 2013).

Allantoin seems to further stimulate tissue growth and support wound healing (Wishart et al., 2022). It was further observed that in rice plants, allantoin supported growth when nitrogen was limited (Redillas et al., 2019). Based on these trends, reduced allantoin levels in S. cunctatrix might negatively affect growth and tissue integrity. This is in accordance with the patterns observed in situ, where S. cunctatrix indeed showed impaired body surface and signs of tissue necrosis (see Chapter 2).

The metabolome adjustments observed in S. cunctatrix specimens living at the vent site, may suggest an overall reduced fitness, based on the physiological consequences correlated to several metabolic pathways. Some degree of osmolytic stress could be anticipated, due to reduced levels of osmoregulatory taurine and trigonelline, and compensatory increases of betaine. Reductions in creatine and ATP levels, also suggest interferences in energetic homeostasis, which can bring about negative impacts on reproduction and filter-feeding activity (reduced flagellar motility). Finally, the overall accumulation of amino acids in vent acclimatized sponges likely reflected a stress status, promoting protein degradation under challenging acidified conditions.
PERSPECTIVES IN METABOLOMICS STUDIES IN SPONGE HOLOBIONTS AND THEIR ENVIRONMENT

In this study we first predicted possible NMR variable candidates, which are likely to be discriminant across sampling sites, where sponge samples live. To distinguish metabolic trends, a multivariate prediction modelling based on OPLS-DA was used. However, care has to be taken, since models are predictions with variable predictive accuracies. Nevertheless, univariate statistical testing validated the significance of metabolite shifts in *S. cunctatrix*. Our results supported that the approximation and fit of data derived from the multivariate modelling actually reflected biologically relevant and discriminant metabolites.

Although the NMR-derived metabolite identification is reproducible and unique, the combination with a complementary MS spectrometry would increase accuracy for metabolite identification (Pan and Raftery, 2007). Especially for apolar fractions, which have not been target in this study, LC-MS based lipidomics analyses could reveal trends in metabolic dynamics across vent and control pH samples. Furthermore, only assigned NMR variables were discussed in this thesis chapter, and it is very likely, that the natural complexity of sponge responses to OA includes more compounds which have not been detected yet.

This chapter underlined species-specific biochemical responses with conceivable physiological interpretations. While in the HMA sponge *C. reniformis* no significant changes in metabolite levels have been revealed, diverse metabolic unbalances have been identified in the LMA sponge *S. cunctatrix*. Several mechanisms in this last species could have contributed to a lowered presumed organismal performance at the vent site, such as increased osmolytic stress, impaired biosynthesis pathways and growth, as well as lower energetic homeostasis. Future studies should complement these results. It would be interesting to determine what is the level of affection in the homeostatic primary versus the secondary metabolism under acidified conditions, analysing full NMR and LC-MS spectra. Also detect whether these potential biochemical changes may derive from variations of the host cells or the microbiome, will be complementary. Such hypotheses could be addressed with stable isotope analysis on separate compartments of host and symbiont cells for total C, N or other atom-type compound contributions, pulse chase experiments and specific marked compound approaches. All and all, an ultimate objective for understanding sponge acclimatization is to correlate responses of host or certain microbial associates to modified metabolic pathways in holobionts inhabiting CO₂ vent sites. But uttermost, other than explaining what is happening inside the host, it would be decisive to describe the contribution of Porifera holobionts in the biochemistry of the surrounding environments. This can be done by analysing their metabolite turn-over resulting from the filtering activities, so between the inhaled and the exhaled seawater.
APPENDIX

Raw data is provided via the following online repository link - CHAPTER III:
https://drive.google.com/drive/folders/1uRAXx6tEs5SWHEjNdxs1Gj6S_6wcdAVO?usp=share_link.
The file “NMR_raw.xlsx” reports relative bin intensities for all NMR variables per sample based on
NMR measurements using polar extracts, which was used for OPLS-DA. The file “metabolites.txt”
contains assigned metabolites with their corresponding relative abundance values per sample.
This data matrix was used for statistical testing (T-Test) and graphical visualization in R Studio.
Metadata are provided in “sample-metadata.txt”.


CHAPTER IV

DEVELOPMENT OF A NEW IN SITU

SAMPLING METHODOLOGY FOR THE

COLLECTION AND STUDY OF FILTER

FEEDING ORGANISMS’ WATER FLUXES
Filter-feeders are critical players in nutrient cycling of benthic ecosystems, but little is known about the specific biomechanisms that take place, and their particular quota in these processes. As a result of organismal metabolic processes during filtering activities, seawater properties are altered, including, particle and nutrient concentrations. Comparing inhaled (In) versus exhaled (Ex) seawater fluxes can reveal which components are being produced or consumed. Current InEx sampling methods require intensive manual work for scuba divers, are prone to contamination by ambient water and receiving insufficient volumes for comprehensive down-stream analyses.

To overcome these limitations, we introduce a submarine peristaltic pump system for an improved InEx sampling procedure. This automated sampling strategy requires only the correct manual positioning of the tubing system into exhalant openings of target animals. With the currently used power supply, the autonomous sampling duration can be extended up to 8 hours. Individually adjustable sampling rates allow to extend this method applicability on diverse filter feeders, with different pumping rates. The pump system is cost-efficient and easy to handle. The low weight of the complete system makes it easily transportable by a diver.

We tested the submarine pump system on the sponge Chondrosia reniformis in laboratory aquaria conditions and in the field (sea rocky communities). Strong reliability in contamination-free sampling of InEx water fluxes was obtained respecting the natural species-specific pumping rates and demonstrated by high bacterial (97%) and planktonic cell removal rates (70-95%) in exhaled seawaters. Using the system, we precisely quantified net production or consumption rates of inorganic and organic nutrients.

The proposed protocol allows to obtain a snap picture of metabolite and nutrient turn-over in filter-feeders in their natural habitat context. Our standardized protocol allows efficient and reproducible InEx sampling and opens up a wide range of experimental opportunities that are suitable for diverse filter-feeders. We discuss how the method may be implemented in a variety of aquatic studies, aiming to study nutrient cycling, filter feeding fluxes, plankton dynamics and seawater metabolomics.

Keywords: InEx water fluxes, filter feeder, Porifera, submarine peristaltic pump, nutrient cycling, marine metabolomics, particle/microbial retention.
INTRODUCTION

MARINE FILTER FEEDERS AND THEIR ECOLOGICAL RELEVANCE

The plethora of ecosystems supplied by marine systems include climate buffering (Reid et al., 2009), coastal protection (Spalding et al., 2014), food provision through fisheries, furnishing of bioactive compounds for drug development (Avila and Angulo-Preckler, 2020; Hayes, 2011; Rocha et al., 2011; Ruocco et al., 2016), transport services, tourism and sport activities, among many others. Our dependence on marine ecosystems and the need to preserve them in good health through sustainable practices are undeniable. Good management plans in relation to our ocean-based activities are of crucial relevance, and to this end we need to fully understand ecosystems functioning.

One keystone functional group of organisms structuring marine communities is constituted by conspicuous, ubiquitous assemblages of suspension (or filter) feeders, mainly represented by sponges, ascidians, bivalve mollusks and crustaceans (Gili and Coma, 1998; Jørgensen, 1975). These organisms are responsible for habitat complexity and shelter provision, but more importantly they are major players in nutrient cycling and bento-pelagic coupling, recycling organic matter and nutrients, which nourishes other life in the water column (Griffiths et al., 2017). By capturing large amounts of plankton, filter feeders directly regulate primary production and indirectly regulate secondary production in littoral food chains (Gili and Coma, 1998).

Sponges (Porifera) in particular, are able to significantly affect seawater biogeochemistry via the so-called ‘Sponge Loop’ (De Goeij et al., 2013). In this process, dissolved organic matter (DOM) is recycled within the sponge, and made available to the surrounding organisms as particulate organic matter (POM), thus fertilizing local communities (Figure 4.1). Considering that DOM molecules are not profitable to most marine organisms, such recycling process allows DOM to re-enter in higher trophic levels, avoiding metabolic/energy losses to the open ocean. Therefore, the study of filter feeders’ activity is essential and timely to estimate the status of our oceans, and to understand how these systems function.

Certain suspension feeders are also being used for bioremediation and for monitoring the health status of marine systems, given their role as major water clearance bio-machineries (Ostroumov, 2005), capable of retaining/accumulating metals (Cid et al., 2010), removing pathogenic microbial agents (Burge et al., 2016), and in some cases biodegrading organic pollutants, e.g. the degradation of polycyclic aromatic hydrocarbons (Marzuki et al., 2021). For these reasons having the capacity
to study particulate and dissolved compounds processed by active suspension feeders is particularly important.

**Figure 4.1: Sponges take up small, non-profitable dissolved nutrients (DOM = Dissolved Organic Matter) which are released as waste products by other benthic organisms including corals and algae. After a nutritional recycling process takes place inside of sponge tissues, a profitable nutrient form (POM = Particulate Organic Matter) is released into the environment. This avoids the loss of nutrients to the open ocean and supports the energy transfer to higher trophic levels, enhancing marine biodiversity.**

The study of filtering activities in marine biotic assemblages is key to address three major processes structuring our oceans: 1) to trace the fate of nutrients, chemicals and contaminants; 2) to reconstruct the vital phenomenon of bento-pelagic coupling that connects living biota and promotes life throughout all depths; and in the end 3) to understand ecosystems functioning and health status. Prior works have focused on analysing filter feeding activity, using varied procedures and equipment. However, the many attempts reported confirm how challenging it is to sample incumbent and excurrent (InEx) fluxes precisely and effectively, especially in situ and in a non-invasive manner.

**Sponges as Case study**

Marine suspension feeders induce water currents to filter out nutrients from the water column. Biological water currents can be induced actively through ciliary or muscular water pumping, as reported in many benthic invertebrates, including bivalves, ascidians, polychaetes and sponges (Jorgenson, 1966; Ludeman et al., 2017). Sponges are very effective sessile filter-feeders and represent an ideal model organism to develop a new InEx sampling method. To supply their nutritional needs, sponges are adapted to filter different kinds of food sources from the water
column, such as suspended particulate and dissolved nutrients (Jiménez and Ribes, 2007; Reiswig, 1971a). Since many sponges inhabit oligotrophic waters, where dissolved nutrients are scarce (Miller, 2009), their filtration system must be particularly efficient to reduce metabolic costs and increase food uptake (Leys et al., 2011). Through their active pumping strategy, sponges are able to filter up to 900 times their body volume of water per hour (Ludeman et al., 2017). Some sponges are able to behaviourally respond to increased water currents to profit from a passive water flow by reducing the volume of water being filtered actively (Leys et al., 2011; Ludeman et al., 2017). Sponges therefore seem to have active control over the volume of water they process and can reduce the energetically expensive filtration process to adapt under stress.

Figure 4.2: Water flow through the aquiferous system of sponges. A) The sponge surface is covered with the inhaling openings ostia. Water flows through canals to choanocyte chambers, where it is filtered and released via excurrent canals into the atrium and then be exhaled through the osculum. Associated microorganisms are widely inhabiting the sponge body, including the body surface and the mesohyl. B) Enlarged view of choanocyte chambers. Water enters the prosopyle and leaves through large exits, the apopyle. C) Enlarged view of the flagellated choanocytes, inducing waterflow through flagellar movement and capturing food particles through a glycocalyx mesh. The glycocalyx forms a web that connects all collars of the choanocyte cells. Amoebocytes phagocytose, transport and release of captured food particles. (Picture adapted from Ludeman et al., 2017).

Their aquiferous system is illustrated in Figure 4.2, indicating the path of filtered water through the sponge body plan. From an outer membrane, that has minute holes (ostia) seawater enters the sponge aquiferous system and is transported via incurrent, branching canals throughout the sponge body towards the choanocyte chambers. These water filtration chambers are provided with a sheet
of tissue to form the entry surface of the chamber, perforated with little holes – the prosopyles (2–3 mm diameter), where the water must pass through. To reach the center of these chambers, the water must further pass a second tissue layer, by passing through collar slits (~0.12 µm diameter). These microvilli collar have a glycocalyx mesh with fine pore sizes of approximately 20×70 nm, which represent the smallest passage in the sponge body (Leys et al., 2011). The cross-sectional area of the choanocyte chambers is expansively increased compared to prior canals, allowing to slow down the water flow and increase the time for filtration processes, where nutrients are absorbed. The choanocyte cells appear to be the primary site for the assimilation of DOM and POM, via pinocytosis and phagocytosis, respectively. Additionally, microbial symbionts are actively involved in DOM uptake (Hudspith et al., 2021). After the filtration process, seawater exits the chambers through the large exit – the apopyle – and enters smaller excurrent canals, which allow to jet the water out into larger excurrent canals, enter the atrium and then be exhaled through the sponge osculum (Leys et al., 2011; Ludeman et al., 2017).

The intense filtering activity constantly exposes sponges to microorganisms present in the seawater, which are either digested by phagocytosis constituting one of their primary food source, or be integrated into the sponge tissue as symbionts (Pita et al., 2018; Sipkema et al., 2015; Wehrl et al., 2007). In the mesohyl of the sponge tissue, carbon- and nitrogen-fixing microbial symbionts can comprise up to 35% of the sponge biomass (Hentschel et al., 2012; Rützler, 1981), while transient microorganisms are mostly present on their body surface (Webster and Thomas, 2016). Microbial symbionts appear to participate in DOM and metabolite uptake, ultimately changing the chemical composition of seawater in the filtration processes (Olinger et al., 2021).

**STATE-OF-THE-ART**

Henry Reiswig et al. pioneered in 1971 the use of in situ techniques for the study of sponge pumping rates and their feeding behaviour (Reiswig, 1971a; Reiswig, 1971b). He developed and applied the first InEx sampling methods to study the particle feeding behaviour of three giant Carribean demosponges. The term InEx stands for the simultaneous, pairwise collection of the water inhaled and exhaled by the filter-feeders. To perform this first sampling approach, Reiswig developed several sampling strategies. For the seawater samples aimed at chemically characterizing particulate organic carbon he positioned a 6 L stoppered bag with a tripod above a sponge and inserted the sampling bag closure into an oscular opening. After removing the stopper, the bag was inflated, capped while still in the exhalant stream and transported to the laboratory, where he quantified particles and compared the results to seawater simply collected near the sponge inhalant tissues (Reiswig, 1971a). For smaller scale microscopic analysis of plankton, he collected exhalant seawater by inserting 25 ml polyethylene syringes with caps into the large sponge oscula.
After removing the caps, syringes were slowly filled while being positioned in the stream. The inhaled seawater samples were collected near the inhalant specimens (Reiswig, 1971a). Furthermore, Reiswig measured for the first time exhalant currents with a battery-powered current recorder equipped with a thermistor sensor (Figure 4.3; FigureMcCammon, 1965), where he observed a variety of pumping patterns among different sponge species, which vary little at the species level (Reiswig, 1971b). This work underlined the necessity of measuring individual sponge pumping activities prior to exhalent water sampling.

Follow-up publications samples InEx water fluxes based Reiswig’s work. Figure 4.4 shows a passive water collection in open-ended polystyrene sterilin pipettes, which were manually aligned as close as possible to exhalent orifices. The exhalent water jet flushed the tubes and replaced the water within. To determine the necessary sampling time, tubes prefilled with fluorescein dyed seawater were flushed with the exhalent jet. The recorded time was multiplied by the factor of 1.5. The inhaled water sampling required additional suction by a syringe attached to one end of the applied pipette tubes. Sampling was ended by closing the tubes with chopped Eppendorf or Tygon tubes as stoppers (Yahel et al., 2005).

Figure 4.3: Schematic representation of Reiswig’s current recorder measuring sponge exhalant water fluxes based on thermistor sensors (Reiswig, 1971b).
The success of clean exhaled water sampling was determined by comparing the concentrations of ultra-planktonic prey taxa, such as *Synechococcus* spp., *Prochlorococcus* spp., picoeukaryotes and heterotrophic bacteria (Yahel et al., 1998). Filter-feeders are known to feed on phytoplankton particles (Reiswig, 1971b; Reiswig, 1974). High cell removal rates in exhaled water samples indicated that sampling was performed cleanly without ‘contamination’ by ambient, non-filtered water (Yahel et al., 2005).

Two years later, the first ROV equipped with a SIP water sampler went down to 120-160m to sample InEx seawater from the glass sponge sponge species is *Aphrocallistes vastus* (Figure 4.5). The new sampler relied on a passive water inflow from an inlet PEEK tubing (external diameter: ~1.59 mm; internal diameter: ~510 µm) into stainless-steel sample cylinders. The process was driven by an external pressure difference of 1500 kPa. Contamination was avoided by staying over an order of magnitude below the excurrent flow from the sponges. As a consequence, sampling was performed with a rate of 1 mL min⁻¹ yielding a total volume of 150 mL per cylinder. One ROPOS manipulator positioned one inlet within the sponge osculum and another one next to the inhalant specimen. Before a second ROPOS arm opened a valve to start sampling, pumping activity was visualized by squirting fluorescein dye at the sponge base (Yahel et al., 2007).
More recent advances for InEx water sampling implied a vacuum-driven technique. The so called VacuSIP is based on a PEEK-tubing which is placed into the exhalent orifices of target filter-feeders using custom-built manipulators. After connecting the tubing to pre-vacuumed sampling vessels, the exhalent, as well as the inhalant seawater, were simultaneously collected, driven by differential pressure differences between the surrounding seawater and the vacuumed glass containers. The sampling rate was adjusted using different sizes of pre-vacuumed sampling containers and tubing length. Analogously to prior protocols, cell counting of prey taxa was used to confirm clean InEx water sampling (Morganti et al., 2016).

One of the main limitations of the VacuSIP is the decreasing sampling rate in time. The increasing volume of sampled water in the containers reduces the vacuum suction force and results in a drop of sampling rates. As a consequence, the sampling of higher volumes requires the change of several containers to maintain the vacuum-suction force, making this sampling method time-intensive and laborous. This sampling technique can only be useful if one is interested in collecting small amounts of seawater. But, comprehensive downstream analysis require larger volumes. To achieve this, a continuous and stable sampling rate is necessary. Another risk of the VacuSIP technique is the ‘contamination’ with surrounding water particularly at the beginning of sampling when a strong suction-force is likely to exceed the natural organismal sampling rate due to the initial vacuum force working at its maximum efficiency. As a rule, sampling rates should be kept low throughout the whole sampling duration to respect a recommended 1% of the original sponge pumping rate (Morganti et al., 2016). This leads to a slow sampling procedure and allows the collection of only small volumes.

Recently, Olinger et al. sampled InEx seawater with a 50 mL syringe manually for subsequent metabolomics analyses. Two divers collected paired 1 L incurrent and excurrent seawater samples. The system implied a polycarbonate check valve attached to the syringes and a teflon tubing which guided the water flow into a 1L foil sample bag (Olinger et al., 2021). However, this very simple sampling procedure was only possible because the target species was the giant barrel sponge (with
individuals reaching diameters of up to 2.5 m (McClain et al., 2015) *Xestospongia muta*, characterized by huge oscula (~0.5 to up to 46.05 cm diameter) and very high pumping rates (McMurray, Blum and Pawlik 2008).

The PeriSIP technique presented in this work proposes a precise and adjustable InEx seawater sampling of higher volumes and sampling rates, targeting filter-feeders with a wide range of exhalent apertures sizes (diameters reaching down to ~5 mm). To target this challenge, a submarine peristaltic pump system with multiple tubing is introduced in combination with custom-built manipulators.

PeriSIP provides a novel efficient InEx in situ nonintrusive, non-invasive method to measure filter feeders activities in situ and in aquaria, in relation to: particle (particulate organic matter, POM) and cell (plankton and microbial cells) removal efficiencies, filtration and feeding rates of inorganic and organic (DOM, dissolved organics matter) nutrients, and nutrition and metabolism (in terms of biomolecule transformation).

**STATEMENT OF CONTRIBUTION**

The underwater peristaltic pump was developed and designed in collaboration with Dr. Volker Meyer, who physically built the device in the electronics workshop of the Max-Planck Institute for Marine Microbiology in Bremen. Fluorescence-activated cell counting (FACS) was performed by Dr. Raffaella Cassotti, at the SZN.
MATERIAL AND METHODS

THE SAMPLING DEVICE – ‘PeriSIP’

Figure 4.6: The PeriSIP sampling device in aquarium set-up. The main components of the submarine pump system are the peristaltic pump, the custom-built manipulator and the underwater battery with three adjustable velocities. Sampled seawater flows from the sponge excurrent opening into the sampling bag (urine bag). Here only one tube is positioned inside of one out of four possible MS/CA cassettes. Picture was taken by Jana Efremova in the aquarium facility at SZN (Napoli, Italy).

A new methodology was developed to precisely collect liquid samples from defined fluid fluxes within a submerged body by using a submarine peristaltic pump system (Figure 4.6A), connected to sampling tubes, a custom-built manipulator, collecting bags and a battery for autonomous power supply. This sampling device assembly has been given the name ‘PeriSIP’. The working principle requires the manual positioning of one or more sampling tubes approximately 1-2 mm inside of a target outflow point, such as a sponge osculum using a manipulator arm (Figure 4.6B). To facilitate the insertion of a larger tube into small inhalant/exhalant outflow pores (e.g., sponge oscula), a plastic tube connector can be integrated with a smaller tip than the diameter of the orifice. Plastic tube connectors can be purchased at different sizes to fit the target InEx sampling source. The sampling tube is embedded inside of an Ismatec MS/CA pressure lever cassette cartridge, which sits on top of the peristaltic pump rotor. The cyclic rotational movement of the rotor volume
displacement through tube compression, and the flow generated leads sampled fluid (e.g., seawater) into the collecting bag – a pressure balanced, inflatable urine bag. The underwater battery is operable at three different voltages, resulting in three different rotational speeds of the pump rotor.

Materials used for the sampling tubes can be purchased depending on the applications. Silicone tubes were used for pilot studies in aquarium conditions, while Tygon tubing was used for final experiments in aquarium conditions and in situ, in natural benthic sponge assemblages. The custom-built manipulator was adapted from (Morganti et al., 2016). A JOBY GorillaPod 5K was attached to a diving weight using a two-component epoxy resin. The pump body and rotor (Figure 4.7A) were designed and 3D-printed in collaboration with Dr. Volker Meyer from the electronics workshop of the Max-Planck-Institute (MPI) for Marine Microbiology in Bremen (Germany). The design gives the possibility to position up to four MS/CA cassettes on top of the rotor (Figure 4.7B), which allows to sample simultaneously from four sampling tubes. The underwater battery and cables were provided by MPI.

Figure 4.7: Submarine peristaltic pump rotor. A) 3D printed rotor of the peristaltic pump with electronics. B) Demonstration of simultaneously using four MS/CA cassettes to sample simultaneously with four tubes.

Collecting bags were sterile 2L urine bags from AIESI® HOSPIDRAIN. These urine bags are provided with a tube, which was cut and connected via Luer adapters to the sampling tubes, including an intermediate adapter system, allowing a washing mechanism of the tubing system (Figure 4.8). The intermediate adapter system is a three-way stopcock, which allows to discard residual untargeted ambient seawater from the tube systems, that could have entered during system assembly underwater. There are two main positions: As displayed in Figure 4.8A, air and ‘contaminating’ environmental water is removed from the system by guiding the flow into the environment. By rotating the Luer-Lock to the position shown in Figure 4.8B, the flow path leads the water samples into the urine bags. The duration for the washing step should be estimated depending on the sampling tube internal volumes and the pumping rates.
**Figure 4.8: Intermediate adapter system (three-way stopcock) between sampling tube and sampling bags.** For clean water sampling, position A is used to discard sampled seawater into the environment. After sufficient rinsing, position B allows a flow of the water sample into the sampling bag. Red arrows symbolize the water flow.

**PROPOSED SAMPLING PROCEDURE AND WORKFLOW**

The proposed sampling procedure for *in situ* studies of filter-feeder water fluxes (Figure 4.9) is divided into 3 phases: 1) The first phase is a pilot study phase, where sampling design and parameters are determined. This is usually easier in aquarium conditions, if the target filter-feeder can be kept in aquarium conditions. During this phase it is important to understand target flux velocities to choose the adequate sampling tube sizes and battery settings for optimal sampling rates. 2) During the second phase, targeted seawater sampling is performed by a scuba diver. 3) After successful sampling water samples are divided and distributed across laboratory-based downstream analyses including quality control. In this step we can guarantee clean water sampling.
Pilot Study

A pilot study should be performed to define a sampling design based on the research question and the target water flux of interest, which requires a pointed seawater sampling technique. For the study of inhaled versus exhaled water fluxes from filter-feeders such as sponges, one possible sampling design is the collection of seawater near the inhalant tissue (ostia) and precisely inside the functional pores (oscula) where the processed water is released back into the environment. The PeriSIP allows to collect water simultaneously from four independent sampling points, allowing sampling designs with more than one filter-feeder, where pairwise comparison or 3-to-1 comparison are of interest. For InEx studies this allows to compare for each sponge one inhaled to one exhaled water sample, but it could be also possible to sample exhaled water from three independent oscula and compare these to one inhaled ambient water sample as control. All four seawater samples are sampled independently in four separated urine bags and can be individually compared to each other.

Before sampling, one important consideration is to estimate target flux velocities such as a species-specific sponge pumping rate. Fluorescein dye (fluorescein, 100 mg L⁻¹) can be used to visualize the exhalant jet and sponge pumping rates are determined using the Fluo-Dye-Front-Method (Figure 4.10). A transparent tube is prefilled with dyed seawater and aligned as close as possible and
perpendicular to an osculum. We used 5 mL Sterilin pipette tubes, often used in cell culture laboratories, in which we cut off the ends and labelled a starting and end point with tape. While closing the distal tube end with a finger, a small amount of fluorescein dye was added into the proximal end of the tube. The tube end is positioned as close as possible to the sponge osculum without touching the sponge. After releasing the finger, the distal end of the tube is open, allowing the exhalant jet coming from the osculum to displace the dyed seawater. The time required to flush the pre-defined volume within the tube is videotaped and pumping rates can be calculated (Yahel et al., 2005).

Figure 4.10: The Fluo-Dye-Speed-Method for the calculation of sponge pumping rates. The displacement of fluorescein dyed seawater was videotaped (n = 5) for five individuals of the sponge *Chondrosia reniformis* with similar oscula diameters. Based on the time that the dyed seawater needed to cross from the starting mark until the end mark, pumping rates were calculated.

Based on the species-specific pumping rates, the sampling velocities of the peristaltic pump are recommended to be kept at maximum 10% of the original flux rate. This guarantees ‘clean’ water sampling of the target water jet, minimizing the risk of ‘contamination’ with surrounding ambient water due to too high suction forces. Additionally, it has to be considered that in case of filter-feeders, large body sizes and oscula increase individual pumping rates and allow faster InEx water sampling (Morganti et al., 2019). Sampling rates of the PeriSIP can be optimized by selecting sampling tubes with a suitable internal diameter of the sampling tubes. Tube length will have no influence on sampling rates and increases flexibility under water to reach spatially distant filter-feeders. Additional fine-tuning of sampling rates is possible by selecting between 3 possible pump
rotor velocities via the velocity settings of the pump battery. Final InEx sampling in aquarium conditions was conducted on 5 different individuals of *C. reniformis* in aquarium conditions with mean sampling rates of 5 mL min\(^{-1}\) (Velocity setting 3, tube with ID 2.7 mm). All sampling equipment was cleaned with a 10% HCl solution and rinsed with double-distilled water between the experiments.

**Sampling Procedure (in situ)**

The *in situ* sampling of filter-feeders’ InEx fluxes is best performed at sampling sites characterized by calm waters with few currents and waves, such as reef systems situated in bays. The selected study site for the PeriSIP was a rocky reef with conspicuous *C. reniformis* assemblages (white arrow in Figure 4.11) at ‘Schiacchetiello’ in the Bay of Naples (40°47’35”N, 14°05’13”E). InEx samples were obtained from *C. reniformis* (n = 6) by snorkelling at 1-2 m depth, using same pump parameters as derived from aquarium testing.

![Sampling Site for in situ InEx sampling of *C. reniformis* water fluxes using the PeriSIP.](image)

**Figure 4.11:** Sampling Site for *in situ* InEx sampling of *C. reniformis* water fluxes using the PeriSIP.

After localizing the target InEx sampling points, the PeriSIP was deployed and assembled next to the study filter-feeders. In case of sponges, it is recommended to test for the presence of individual fluxes by applying fluorescein next to inhalant orifices and visually detect water displacement (qualitative check).

The correct assembly of the PeriSIP is shown in Figure 4.12. First, the manipulator is placed close to the sponge so that the sampling tubes can reach the osculum. If necessary, additional diving weights can be used to further stabilize the manipulator. Without touching the sponge, proximal ends of the sampling tubes are inserted inside of exhalant orifices using the flexible manipulator arms. This
step requires a certain degree of manual skills, therefore prior training in aquarium conditions are highly recommended to avoid organismal disturbance and consequent sampling interferences.

Figure 4.12: Submarine peristaltic pump system deployed in situ near a natural rocky reef community of the sponge *C. reniformis*. **A** Peristaltic pump system including 1: sampling bag 2: pump 3: battery with 3 velocities 4: sponge 5: manipulator 6: sampling tube **B** enlarged photo of the three-way stopcock, here: sampling position **C** enlarged photo of *C. reniformis* and sampling tube positions for 1: “In” sampling tube next to inhalant specimen and 2: “Ex” sampling tube positioned inside the exhalant osculum (Photography by Alberto Colletti).

After successful installation of the PeriSIP tubing, the pump can be started by rotating the speed controller to the desired velocity setting for pump rotation. The three-way stopcock adapter, introduced between sampling tubes and the inflatable sampling bag, should be first positioned according to Figure 4.8A, so that seawater is not sampled into the urine bags, but released into the environment. This allows to flush the tubes and removes all residing air bubbles and seawater inside of the sampling tubes. After washing the tubes, the adapter system is turned manually by the diver to start sampling into the sampling bags. The duration of this washing step must be chosen depending on tube length and volume. Sampling rates can be modified under water, selecting
between the 3 different velocity settings of the battery. After the desired volume is sampled, filled sampling bags are easily removed and transported in cooling boxes to the laboratory for downstream analyses. All sampling equipment was cleaned with a 10% HCl solution prior sampling. One consideration for the choice of manipulator systems is the geophysical property of the reef. Sponges inhabiting vertical walls (Figure 4.13), can be targeted if irregular rocks and cavities allow to fix the manipulators. We used two different kinds of manipulators (Figure 4.13A). One attached to a simple diving weight showed better fit into cavities with a horizontally flat level (Figure 4.13A-2a). The second system was created by connecting two tripods to each other using epoxy resin and wrap a flexible weight around the manipulator centre (Figure 4.13A-2b). This way the bottom manipulator was tightly attached to irregular rocks and reaching its arms into small cavities, while the upper manipulator served to hold the sampling tubes in position.

Figure 4.13: PeriSIP installation at vertical reef walls. A-D) show the positioning of weight-stabilized manipulators at a vertical reef wall and PeriSIP deployment. A) Two kind of manipulators were used depending on reef properties. B) The installation of the sampling tube inside of the sponge osculum. D) The battery deployment on the sediment is facilitated by using a long cable. Abbreviations: 1: Sponge (Chondrosia reniformis) 2: manipulator 3: pump 4: sampling bag 5: battery (Photos by Jana Efremova).
Quality control and downstream analyses (Laboratory)

Quality control using flow cytometry of InEx samples is important to justify further downstream analyses and allows the study of plankton communities in InEx fluxes. Inhaled and exhaled samples should differ markedly in phytoplankton concentrations, based on filter-feeders retaining certain planktonic cells to supply their nutritional needs (Morganti et al., 2016; Reiswig, 1971a). Further downstream analyses can then proceed, such as inorganic or organic nutrient analyses, the identification of allelochemicals and metabolites, or the quantification of heavy metals and more, depending on the research question of interest.

Quality control

The quality of InEx samples were checked based on the retention of picoplanktonic cells (autotrophic and heterotrophic, both prokaryotes and eukaryotes) using flow cytometry. Cell concentrations were expected to be lower in exhaled samples, due to cell retention during filter-feeding. For *C. reniformis*, typical retention efficiency should result at approximately 50–70% for typical dietary prey taxa, such as *Synechococcus* spp. (Morganti et al., 2016). Retention efficiencies vary depending on the filter-feeding species (Mueller et al., 2014; Perea-Blázquez et al., 2012a). After confirming species-dependent retention rates for all InEx samples, the sampling procedure was classified as successful and further downstream analyses were performed.

The efficiency of cell retention by the sponge was calculated according to:

\[
\text{Retention efficiency} [\%] = \left( \frac{\text{In} - \text{Ex}}{\text{In}} \right) \times 100\%
\]

where In, Ex are the cell counts (cells/ml) of a given cell type determined by flow cytometry in inhaled and exhaled seawater samples.

Sample processing for this quality check required an aliquot of each InEx seawater sample (1 mL), which was fixed with a mix of paraformaldehyde and glutaraldehyde (1% and 0.01% final concentration, respectively) and directly snap-frozen in dry ice or liquid nitrogen. Upon arrival to the laboratory, samples were stored at -80°C until analyses (Marie et al., 1999). The quantification of picoplanktonic cells (autotrophic and heterotrophic, both prokaryotes and picoeukaryotes) were determined using flow cytometry. Cytogram analysis was conducted using FCS Express software (De Novo Software, Los Angeles, CA). Cytometric clusters included *Synechococcus* spp. and picoeukaryotes, discriminated based on their relative scatter and autofluorescence (orange from phycoerythrin and red from chlorophyll for the first, and red only for the latter). Heterotrophic prokaryotes, which are not autofluorescent, were discriminated from background noise based on their scatter and green fluorescence (from SYBRGreen stain, proxy of DNA content), as in Marie et al., 1999 and Balestra et al., 2011. The flow cytometer used is a Becton Dickinson FACSVerse instrument with standard filter set.
**Downstream Analyses**

Downstream analyses performed on InEx samples of *C. reniformis* aimed at quantifying inorganic and organic nutrients. For inorganic dissolved nitrogen (DIN) analyses seawater was filtered through cellulose acetate membrane filters (pore size: 0.22 μm) into 20 mL high-density polyethylene vials and stored at -20°C until analyses. Measurements were performed for ammonium, nitrate and nitrite, carried out on a five-channel continuous flow autoanalyzer (Flow-Sys Systea), according to Hansen and Grasshoff (1983), modified.

For total organic carbon (TOC) and nitrogen (TN), as well as dissolved organic carbon (DOC), samples were collected into acid washed 30 mL HDPE sample bottles and were directly acidified with HCl 37% (pH ≤ 2) to remove inorganic carbon and stored in the dark. DOC samples were additionally filtered through a pre-combusted GF/F filter (Santinelli, 2015). The analyses were carried out by using a Shimadzu TOC-L analyzer. The reliability of the measurements of the organic carbon (TOC and DOC) was verified daily by comparison with DOC Consensus Reference Waters (Hansell, 2005; Sharp et al., 2002). Potassium phthalate was used as standards. TN was measured simultaneously with DOC or TOC by using the TN unit connected to the Shimadzu TOC-L analyzer. Potassium nitrate was used as standards and the seawater reference material from the Consensus Reference Material Project (CRM; http://yyy.rsmas.miami.edu/groups/biogeochem/CRM.html) was used to determine the precision and accuracy of the method.

**Theoretical Design of a Thermistor Flowmeter**

In collaboration with Dr. Volker Meyer from the MPI Bremen, the design of a thermistor flowmeter was developed, which can be included in the PeriSIP system (Figure 4.14) to measure target flux rates and to autonomically adjust sampling rates accordingly. Next to the sampling tube, a flow-sensor can be positioned inside of the sponge osculum to measure current speeds. The principle for flow measurement is based on the hot bead thermistor flowmeter (LaBarbera and Vogel, 1976), using semiconducting NTC (Negative temperature coefficient of resistance) sensors, which are sensitive to heat. NTC sensors are built from sintered semiconducting ceramic material containing a mixture of several metal oxides. Briefly, one heated NTC (Figure 4.14: red NTC1) is positioned inside of the osculum and measures the flow, while a second NTC (Figure 4.14: blue NTC2) measure the reference ambient temperature. A temperature difference between NTC1 and NTC2 is created by heating NTC1 by usually 1-2°C more than the ambient temperature. As an example, in 25°C seawater temperature, NTC1 is set at 26-27°C. This increase in temperature will not stress the sponge or impact negatively sponge pumping rates. The water flow from the sponge osculum changes the local temperature at NTC1. The energy needed to maintain the original temperature difference between NTC1 and NTC2 are then processed by the Hot-Bead-Flow-Sensor into a current flow.
Figure 4.14: Thermistor flowmeter integration into the PeriSIP system. This schematical diagram shows the working principle and electronics of a flowmeter based on a Hot-Bead-Flow-Sensor with two NTCs. Black arrows indicate electronic flow of information.
The flow data are recorded by a logger and visualized in a display. The adjustable Level-Switch triggers the ON/OFF and speed-control of the pump-battery using the principles of a PWM-Controller (PWM = pulse-width modulation) in dependence of the measured flow rates. Depending on the target filter-feeding organism, a minimum flow threshold can be set before sampling. This will ensure, that the motor is not started unless the target flow speed is present, guaranteeing that the pump is switched off in case the sponge does not pump water. Flux velocities can be measured during the whole sampling procedure to allow real time monitoring of pumping rates. Additionally, the Level-Switch triggers the Speed-Control to allow a higher rotational speed of the peristaltic pump at high flow rates of the sponge and automatically slower rotational speeds at lower flow rates. This allows the application of adjustable sampling rates at optimal efficiency and minimal risk of contamination. Conclusively, the integration of a flow-sensor adjusts sampling rates automatically and in real-time depending on the present filter-feeders exhaled water-flow.

**THE VACUSIP**

To compare and show that the PeriSIP method is advancing existing state-of-the-art methods, we assembled the VacuSIP device (Figure 4.15) according to Morganti et al. (2016) and sampled InEx seawater samples in aquarium conditions from the sponge *C. reniformis* (n = 5). The time required for sampling, the yielded volumes and final cell counts were then compared to InEx samples collected with the PeriSIP.

*Figure 4.15: VacuSIP assembled and tested in aquarium conditions to compare to PeriSIP sampling efficiencies.* A) Shows the pre-vacuumed vial. Upon piercing septum cap with a needle connected to the PEEK tube, water flow started. B) Peek tube positioned inside of an osculum of the sponge *C. reniformis*, using the manipulator arm.
RESULTS

PILOT STUDY

DETERMINATION OF OPTIMAL SAMPLING RATES

Applying the fluo-dye speed method we experimentally determined the sponge-specific pumping rate of 80 mL min⁻¹, i.e. 4.8±0.8 Lh⁻¹ for *C. reniformis* (Figure 4.16A). Based on this result, we defined optimal sampling rates to be within the order of magnitude 1-10% of the sponge-specific pumping rate, i.e. ranging between 0.8 and 8 mL min⁻¹.

After testing several tube diameters, we decided to use a Tygon tubing with an internal diameter of 2.7 mm. Figure 4.16 shows the determined sampling rates using the 3 possible pump rotor velocities, which resulted in sampling rates of 1.9, 3.1 and 5.1 mL min⁻¹, respectively for 3 battery settings (Figure 4.16B). Repetitive sampling with volumetric measurements resulted in very low variabilities of each sampling rate. All rates were measured experimentally in aquarium tests during a period of 5 h of autonomous operating time. For the present case study, we used Velocity 3 to optimize sampling volumes per time.

![Figure 4.16: Sponge pumping rate for *C. reniformis* (A) and experimental pump sampling rates for 3 battery velocities using tube internal diameter of 2.7 mm (B). Pump sampling rates were experimentally measured (n = 5) during a sampling period of 5h in the aquarium facility at SZN (Naples, Italy).](image)

TESTING PeriSIP SAMPLING RATES IN AQUARIUM CONDITIONS

We confirmed in aquarium conditions that all 3 sampling velocities with the selected sampling tube (ID = 2.7 mm) yielded ‘clean’ InEx water sampling of *C. reniformis* water fluxes. Based on measuring high cell counts, we could confirm that Ex samples were not ‘contaminated’ by ambient water, which would increase cell counts in exhaled seawater samples. As shown in Figure 4.17, *Synechococcus* spp. and heterotrophic bacteria were retained at 52±5% and 85±4%, respectively.
Results showed very low variability, confirming stable and contamination free sampling with high reproducibility.

![Graphs showing log Synchococcus per mL and log bacteria per mL](image)

**Figure 4.17**: *Synechococcus* spp. (A) and bacterial cell counts (B) of InEx seawater fluxes collected from the sponge *C. reniformis* in aquarium conditions, sampled with 3 velocities for each inhaled and exhaled sample. The 3 different battery velocities tested on 5 different sponge individuals revealed very low variability in resulting cell counts. Sampling was repeated (n = 3) for each individual and velocity. Boxplots show range (whiskers), median (bold line), and interquartile range (box height) of cell counts. We further confirmed that the PeriSIP device or tubing did not have any influence on final cell counts (Figure 4.18). This was demonstrated by comparing cell counts of ambient aquarium seawater collected with 50 mL falcon flasks to those collected with the PeriSIP, which resulted in no significant difference (according to t-test with p < 0.05).

![Boxplot showing bacterial cell counts](image)

**Figure 4.18**: FACS cell counts from collecting aquarium seawater using 50 mL falcon tubes vs. pump sampling using the PeriSIP (n=5). Boxplots show range (whiskers), median (bold line), and interquartile range (box height) of bacterial cell counts.
AQUARIUM TESTING: PeriSIP versus VacuSIP

The PeriSIP resulted in higher retention rates in the exhaled seawater compared to the VacuSIP, while also maintaining lower variability. Figure 4.19 shows the retention efficiency of *Synechococcus* spp. and bacterial cells after InEx sampling using the state-of-the-art method VacuSIP versus the proposed PeriSIP method. InEx sampling with the PeriSIP resulted in 89.8±0.5% and 98.6±0.1% for *Synechococcus* and bacterial cells, respectively. Instead, sampling with the VacuSIP showed higher cell counts in the exhaled seawater and consequently resulted in lower cell retention with mean rates of 86.9±4.6% and 94.6±0.7% for *Synechococcus* and bacterial cells, respectively. Variability between InEx cell counts was slightly higher for VacuSIP results compared to PeriSIP, but not statistically significant (according to t-test with p < 0.05).

![Graph showing retention efficiency](image)

**Figure 4.19:** *Synechococcus* spp. and bacterial cell counts of *C. reniformis* (n = 5) InEx seawater samples collected with the VacuSIP method and the PeriSIP method. Shown are mean retention efficiencies ± standard deviation.

Comparing the sample volume yield per time, revealed that the PeriSIP allowed the sampling of higher volumes in shorter time (Figure 4.20). The VacuSIP plateaued at maximum 15 mL, using a 40 mL pre-vacuumed vial after approximately 10 min. To obtain higher volumes repeated sampling was required with tube replacement. The sampled volume collected with the PeriSIP increased linearly, thus the resulting volume depended on the set rotational velocity, sampling duration and sampling bag volume. Depending on the battery setting, the PeriSIP yielded sampling rates of approximately 83 mL hr⁻¹, 186 mL hr⁻¹ and 308 mL hr⁻¹ for velocity 1, 2 and 3, respectively.
**IN SITU APPLICATION OF THE PERISIP**

**PLANKTONIC CELL RETENTION BY C. RENIFORMIS**

InEx sampling of *C. reniformis* water fluxes revealed high cell retention rates for phytoplanktonic cells. Based on the cytogram (Figure 4.21A) comparing cell counts of inhaled versus exhaled seawater from *C. reniformis* (n = 6), phytoplanktonic cells were quantified. Based on cell counts, the sponge retained *Synechococcus* spp., picoeukaryotes and *Prochlorococcus* at efficiencies of 86.3±4.6%, 70.2±19.3% and 95.5±3.1%, respectively (Figure 4.21B). Extraordinarily high removal rates with very low variation were observed for heterotrophic bacteria. These were retained at 97.1±3.5% relative to inhaled, i.e. ambient seawater concentrations.
Figure 4.21: Determination of sampling quality by flow cytometry analysis of paired inhaled vs. exhaled water samples collected in situ from the sponge *C. reniformis*. A) Cytogram based on red and green fluorescence showing phytoplankton populations and heterotrophic bacterial cell counts, respectively. B) Quantitative retention efficiency (%) exhaled vs inhaled cell counts per cell type, based on cell counts derived from cytogram analyses. Shown are mean retention efficiencies ± standard deviation.

**NUTRIENT FLUXES IN C. RENIFORMIS: AQUARIUM VERSUS IN SITU**

The concentrations of particulate nutrient sources, including heterotrophic bacteria, *Synechococcus* and *Prochlorococcus* cells, differed substantially comparing *in situ* and aquarium conditions (Table 4.1 and Figure 4.22). *Synechococcus* and *Prochlorococcus* cell counts were higher *in situ* compared to aquarium seawater. *Synechococcus* counts dropped from $6.7 \times 10^6 \pm 2.3 \times 10^6$ cells per mL *in situ* down to $1.3 \times 10^4 \pm 0.6 \times 10^3$ cells per mL in aquaria, representing ~500-times lower concentrations in aquaria. Similarly, *Prochlorococcus* cell counts dropped by a factor of approximately 325 in aquarium conditions, manifested by a decrease from $4.8 \times 10^6 \pm 1.7 \times 10^6$ cells per mL down to $1.4 \times 10^4 \pm 1.2 \times 10^3$ cells per mL. In contrast, cell counts of heterotrophic bacteria were $1.0 \times 10^6 \pm 1.4 \times 10^4$ cells per mL *in aquarium conditions*, versus $4.5 \times 10^5 \pm 7.5 \times 10^4$ cells per mL.
cells per mL in *in situ* conditions, revealing more than two-fold lower concentrations in natural reef conditions.

### Table 4.1: Absolute cell counts of inhaled water samples in aquarium conditions and *in situ*.

<table>
<thead>
<tr>
<th></th>
<th>Bacteria mL⁻¹</th>
<th>sd</th>
<th>Synechococcus mL⁻¹</th>
<th>sd</th>
<th>Prochlorococcus mL⁻¹</th>
<th>sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquarium</td>
<td>1032763</td>
<td>14708</td>
<td>13314</td>
<td>673</td>
<td>14733</td>
<td>1223</td>
</tr>
<tr>
<td><em>in situ</em></td>
<td>451046</td>
<td>75400</td>
<td>6742176</td>
<td>2363292</td>
<td>4800040</td>
<td>1791942</td>
</tr>
</tbody>
</table>

Figure 4.22: Relative comparison of phytoplanktonic cell counts *in situ* and in aquarium conditions. Presented are log-transformed cell counts of inhaled seawater samples from the aquarium facility and collected *in situ* (n = 5 each). Boxplots show range (whiskers), median (bold line), and interquartile range (box height) of cell counts.

Table 4.2 summarizes all measured concentrations of inorganic and organic nitrogen and carbon compounds for InEx seawater fluxes sampled from *C. reniformis* *in situ* (Figure 4.23A) and in aquarium conditions (Figure 4.23B) using the ‘PeriSIP’.

### Table 4.2: Inorganic and organic nutrient concentrations (µM) for *C. reniformis* InEx fluxes (n = 5) *in situ* and in aquarium conditions. ∆ net fluxes positive values (red) represent a net production, while a negative value for Δ fluxes (blue) represent a net consumption.

<table>
<thead>
<tr>
<th></th>
<th>NH₄⁺</th>
<th>sd</th>
<th>NO₂⁻</th>
<th>sd</th>
<th>NO₃⁻</th>
<th>sd</th>
<th>TOC</th>
<th>sd</th>
<th>DOC</th>
<th>sd</th>
<th>TN</th>
<th>sd</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>in situ</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In</td>
<td>1.0</td>
<td>0.2</td>
<td>0.08</td>
<td>0.01</td>
<td>0.7</td>
<td>0.2</td>
<td>185.8</td>
<td>128.8</td>
<td>146.2</td>
<td>76.1</td>
<td>5.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Ex</td>
<td>0.6</td>
<td>0.3</td>
<td>0.05</td>
<td>0.01</td>
<td>2.1</td>
<td>0.4</td>
<td>272.0</td>
<td>112.2</td>
<td>221.4</td>
<td>63.4</td>
<td>6.0</td>
<td>0.7</td>
</tr>
<tr>
<td>∆ (In-Ex)</td>
<td>-0.4</td>
<td>-0.03</td>
<td>1.4</td>
<td>1.4</td>
<td>86.2</td>
<td>75.2</td>
<td>81.3</td>
<td>5.0</td>
<td>21.2</td>
<td>8.0</td>
<td>21.2</td>
<td>5.1</td>
</tr>
<tr>
<td><em>Aquarium</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In</td>
<td>3.4</td>
<td>1.2</td>
<td>0.47</td>
<td>0.02</td>
<td>8.2</td>
<td>1.1</td>
<td>136.7</td>
<td>11.8</td>
<td>105.9</td>
<td>6.1</td>
<td>24.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Ex</td>
<td>2.1</td>
<td>0.4</td>
<td>0.44</td>
<td>0.26</td>
<td>13.0</td>
<td>4.7</td>
<td>89.2</td>
<td>5.0</td>
<td>81.3</td>
<td>8.0</td>
<td>21.2</td>
<td>5.1</td>
</tr>
<tr>
<td>∆ (In-Ex)</td>
<td>-1.3</td>
<td>-0.03</td>
<td>4.8</td>
<td>4.8</td>
<td>-47.5</td>
<td>-24.6</td>
<td>-24.6</td>
<td>-2.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.23: Inorganic and organic nutrient analyses of InEx water sampled of the sponge *C. reniformis* (*n* = 6). Shown are mean concentrations (µM) ± standard deviation.

The analysis of inorganic nitrate compounds of *in situ* InEx water fluxes revealed a decreased concentration for ammonium and nitrite in exhaled seawater of *C. reniformis*. Ammonium levels in inhaled seawater samples had mean concentrations of 0.99 ± 0.15 µM, while exhaled samples showed a lower concentration of 0.63 ± 0.27 µM. Analogously, Nitrite levels dropped from 0.08 ± 0.01 µM down to 0.05 ± 0.01 µM in the exhaled fluxes. Nitrate instead was more concentrated in exhaled samples, 2.10 ± 0.39 µM, than in inhaled seawater 0.69 ± 0.24 µM.

Considering organic nutrients, TN was present at 5.18 ± 0.99 µM in inhaled seawater and slightly increased to 5.96 ± 0.72 µM in exhaled samples. TOC samples showed a stronger increase in exhaled seawater with levels rising from 185.78 ± 128.80 µM in inhaled up to 271.95 ± 112.19 µM in exhaled
seawater samples. DOC analysis showed the same trends with slightly lower concentrations across all InEx samples.

In aquarium conditions, same trends for inorganic nitrogen compounds were observed but with overall increased nutrient concentrations compared to in situ results. Ammonium levels of 3.40 ± 1.22 µM were higher in inhaled samples and decreased to 2.12 ± 0.43 µM in exhaled seawater. Nitrite concentrations were also notably elevated in aquarium conditions, but decreased from 0.47 ±0.02 µM in inhaled down to 0.44 ± 0.26 µM in exhaled samples. Nitrate levels were more than 10-fold higher in aquaria and resulted in mean concentrations of 8.17± 1.11 µM for inhaled and 13.01 ± 4.68 µM for exhaled seawater samples.

TN was almost 5 times more concentrated in aquarium conditions with concentrations of 24.14 ± 1.53 µM for inhaled and 21.19 ± 5.08 µM for exhaled samples. Compared to in situ levels, TOC and DOC reported lower concentrations in aquarium conditions and resulted in 136.68 ± 11.77 µM and 105.87 ± 6.07 µM, respectively. In sponge exhaled seawaters, TOC and DOC levels further decreased down to 89.22 ± 4.97 µM and 81.29 ± 7.96 µM, respectively.
DISCUSSION

The study of water fluxes in filter-feeders is of special ecological relevance. Their capacities to change seawater chemistry, can support nutrient recycling, bento-pelagic coupling and nourish marine life (Griffiths et al., 2017). Especially sponges play a central role in nutrient recycling via the ‘Sponge Loop’ (De Goeij et al., 2013). Besides their ecological relevance, suspension feeders can be applied in bioremediation of fresh and marine environments (Ostroumov, 2005), to remove pollutants such as metals (Cid et al., 2010), pathogenic microbial agents (Burge et al., 2016) and polycyclic aromatic hydrocarbons (Marzuki et al., 2021).

The number of publications comparing the seawater chemistry before and after filtration by a filter-feeder are very scarce. One of the reasons is likely the challenging process of sampling inhaled and exhaled seawater separately, while both fluxes merge in the water body. Sampling methods where exhaled seawater is collected manually using simplified devices, is only possible if target filter-feeders have big exhalant openings. For instance, InEx sampling performed by Reiswig in the early 70s was performed with simple bags or syringes capturing the water fluxes of three carribean demosponges with particularly large exhalant oscula (Reiswig, 1971a). Subsequent studies applying similar simplified sampling methodologies have demonstrated indeed, significant changes in the chemical profiles of the seawater as a result of the filtering activities of giant Caribbean sponges (Fiore et al., 2017; Olinger et al., 2021). An upgraded water sampler was the SIP, developed by Yahel et al. (2007), which was tested on the glass sponge Aphrocallistes vastus, which has again large oscula of up to 8 cm in diameter.

The latest and most advanced InEx sampling method previous to ours was developed in 2016, and was called ‘VacuSIP’ (Morganti et al., 2016). This device was used on C. reniformis and allowed for the first time the sampling of water fluxes from sponges with smaller oscula, ranging between 2 and 5 mm of diameter. This apparatus was specifically designed to be applied in Porifera, to collect a limited volume of seawater, depending on the capacity of pre-vacuumed collecting tubes. However, the ‘VacuSIP’ has a number of limitations that are resolved with the hereby proposed sampling procedure ‘PeriSIP’, based on a submarine peristaltic pump system. In the next sections we demonstrate the flaws of ‘VacuSIP’ and other InEx fluxes sampling devices compared to the new sampling procedure invention, while exposing the competetive advantages and potential ecological and industrial applicabilities of ‘PeriSIP’.
COMPETITIVE ADVANTAGES OF THE PERISIP METHOD

The ‘PeriSIP’ system provides a precise and adjustable InEx seawater sampling, using constant sampling rates and allowing the collection of large fluid volumes. To achieve this, a submarine peristaltic pump based system is assembled. In Table 4.3, we highlight the main advantages of the new ‘PeriSIP’ system compared to the most advanced method ‘VacuSIP’. Sample quality was assessed by comparing species-specific retention rates of *C. reniformis* (Morganti et al., 2016).

Table 4.3: Comparison of the most upgraded InEx sampling technique ‘VacuSIP’ and our introduced ‘PeriSIP’. Sampling conditions are marked with * aquarium ** in situ. [1] in 20 mL pre-vacuumed vials (testing in SZN aquarium facility); [2] Morganti reports 500 mL in 1 L glass bottles (could not be confirmed during our testing); [3] Sampling for 8 hours at a rate of 5.1 mL min⁻¹.

<table>
<thead>
<tr>
<th>Sample Quality</th>
<th>VacuSIP</th>
<th>PeriSIP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell retention:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In versus Ex</td>
<td>Heterotrophic bacteria</td>
<td>94.6±0.7%*</td>
</tr>
<tr>
<td><em>(C. reniformis)</em></td>
<td></td>
<td>97.1±3.5%**</td>
</tr>
<tr>
<td>Synechococcus</td>
<td>86.9±4.6%*</td>
<td>97.5±0.6%*</td>
</tr>
<tr>
<td></td>
<td>~48%**</td>
<td>89.8±0.5%*</td>
</tr>
<tr>
<td><em>(Morganti et al., 2016)</em></td>
<td></td>
<td>86.3±4.6%**</td>
</tr>
<tr>
<td>Prochlorococcus</td>
<td>96.5±3.1%*</td>
<td>97.5±0.6%*</td>
</tr>
<tr>
<td></td>
<td>~7%**</td>
<td>70.2±19.3%**</td>
</tr>
<tr>
<td><em>(Morganti et al., 2016)</em></td>
<td></td>
<td><em>(Morganti et al., 2016)</em></td>
</tr>
<tr>
<td>Picoeukaryotes</td>
<td>~70%**</td>
<td>70.2±19.3%**</td>
</tr>
<tr>
<td><em>(Morganti et al., 2016)</em></td>
<td></td>
<td><em>(Morganti et al., 2016)</em></td>
</tr>
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</table>

**Effectiveness of sampling**

<table>
<thead>
<tr>
<th></th>
<th>VacuSIP</th>
<th>PeriSIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vacuum required</td>
<td>✓</td>
<td>✘</td>
</tr>
<tr>
<td>Sampling rate</td>
<td>constant</td>
<td>✘</td>
</tr>
<tr>
<td></td>
<td>adjustable</td>
<td>✘</td>
</tr>
<tr>
<td>Adjustment of sampling rate by</td>
<td>Tube-length</td>
<td>Tube ID and rotor velocity</td>
</tr>
<tr>
<td>Maximum operating time</td>
<td>Vacuum dependent <em>(Frequent vial changes necessary)</em></td>
<td>8 hours</td>
</tr>
</tbody>
</table>

**Effort by the involved personnel**

<table>
<thead>
<tr>
<th></th>
<th>VacuSIP</th>
<th>PeriSIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Easy handling</td>
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<td>✓</td>
</tr>
<tr>
<td>Power supply needed</td>
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<td>✓</td>
</tr>
<tr>
<td>Cost (Euro, approx.)</td>
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<td>1000</td>
</tr>
</tbody>
</table>
SAMPLE QUALITY

One big advantage of ‘PeriSIP’ over ‘VacuSIP’ is the increased sample quality due to minimized contamination by ambient seawater, resulting in higher cell retention rates. Contamination in this case is the unwanted collection of ambient water together with the exhaled seawater of the filter-feeding organism. A successful InEx sampling method scrutinizes total separation of exhaled seawater from ambient water. Cell retention rates were determined by comparing cell counts of typical filter feeder dietary prey taxa from the exhaled with respect to the inhaled samples. Direct comparisons of aquarium testing indicated that the PeriSIP generally resulted in higher cell retention rates and lower inter-sample variability compared to the VacuSIP. This is likely due to a more stable and continuous sampling procedure in PeriSIP, through a constant rotational speed of the peristaltic pump rotor, causing a constant water flow. Instead, VacuSIP depends on the vacuum-suction force created in the pre-vacuumed vials. This further explains the higher risk of contaminating samples with surrounding water with ‘VacuSIP’, particularly at the beginning of sampling when the intense initial suction-force is likely to exceed the recommended sampling rate. The stable sampling parameters of the PeriSIP thus represent a clear improvement and allows a cleaner separation of water samples. This will further influence the quality of following downstream analyses and will allow to obtain a more realistic picture of the ecological context of InEx fluxes. After testing the PeriSIP in situ, results further underlined the robustness of the sampling strategy by showing very similar cell retention rates, whereas the ‘VacuSIP’ reported in situ distinctly lower cell retention (Supplementary, Morganti et al., 2016). This further might be related to more intricate manipulations (e.g., periodic tube replacements) involved in the handling with VacuSIP, likely increasing the risk for contamination.

Retention rates can vary between different filter-feeding species. Mueller et al. (2014) used the VacuSIP to sample InEx fluxes from the two coral-excavating sponges Siphonodictyon spp. and Cliona delitrix, and reported diversified bacterial cell retention rates of 72±15% and 87±10%, respectively. Conclusively, prior pilot studies in aquarium conditions are helpful to define a species-specific threshold for cell retention rates and optimize the collection of in situ InEx samples.

EFFECTIVENESS OF SAMPLING

One of the main limitations of the VacuSIP is the decreasing sampling flow over time. The increasing volume of sampled water in the containers reduces the vacuum suction force and results in a drop of the sampling rate. As a consequence, the sampling of larger volumes requires a continuous replacement of pre-vacuumed containers to maintain the vacuum-suction force, making this sampling method time-intensive and laborous. Overall sampling rates have to be kept low throughout the whole sampling duration to respect the recommended 1–10% of the original sponge pumping rate (Morganti et al., 2016). Using stronger vacuum to increase sampling rates
would increase the risk of contamination at the beginning when the suction-force is at its maximum. The ‘VacuSIP’ technique can be useful if one is interested in collecting small amounts of seawater. But, comprehensive downstream analysis require higher volumes. To achieve this, a continuous and stable sampling rate is necessary.

The ‘PeriSIP’ improves the current sampling methods with its continuous pumping rates. This allows to adapt InEx seawater sampling to a wide range of different filter-feeding species and optimizes the yield of water collection over time. The versatile sampling rate adjustment allows to collect efficiently InEx fluxes from filter-feeders with slow pumping rates and small oscula that were not possible with previous devices. Key of sampling with the ‘PeriSIP’ is that the seawater can be sampled at higher rates, i.e. up to 10% of the original filter-feeder pumping rate. The device is operable at 3 different pumping velocities, which can be easily switched underwater, allowing to perform samplings with diverse characteristics and purposes in a single dive. In addition, sampling rates can be additionally adjusted by introducing tubes with different internal diameters.

VacuSIP technique in lieu, can adjust for differing sampling rates by the length of the PEEK-tubes and the vacuum force created in the vials, this last depending on the size of the tubes. Another factor to be considered when using VacuSIP is depth. At increasing depth, the more elevated external pressure accelerates the sampling rate in any vacuum-based device. The length of the PEEK tubing must be calculated prior to sampling, to define an estimated rate at a precise depth. Indeed, sampling in aquarium conditions is limited when using VacuSIP, as at minimal atmospheric pressure sampling becomes extremely slow. The PeriSIP works depth-independent, making the application of the pump versatile across different depths during one dive, other than suitable for aquaria approaches.

After a stable installation, the PeriSIP allows an autonomous sampling of up to 8 hours, without the need to be monitored by a diver. This allows to sample higher volumes even when very slow sampling rates are required. Logistically this is of extreme importance, since a scuba diver can stay maximum for approximately 90 minutes under water. Two dives are sufficient to first deploy the apparatus and then to collect the samples after the desired volume is collected.

Larger sample volumes allow to perform a higher number of downstream analyses of InEx water fluxes (Figure 4.24). Besides the FACS, organic and inorganic nutrient analyses, that have already been possible with the VacuSIP, future studies could use the PeriSIP sampling to perform, for instance, chemical and metabolomics profiling and DOM characterization of InEx fluxes. Depending on the protocol, metabolomics approaches require between 100 and 1000 mL of seawater (Dittmar et al., 2008; Petras et al., 2017). Microbiome studies can be targeted, which usually require a minimum of 1 L of seawater to be filtered on 0.22 µM membranes to accumulate microbial communities for sequencing (e.g. Röthig et al., 2017). Dissolved gas measurements can further be performed by membrane inlet mass spectrometry (MIMS) (Davey et al., 2011; Tortell, 2005), which
cannot be performed with VacuSIP collected seawater samples. Upon collecting seawater into pre-vacuumed vials, a headspace persists in which gases equilibrate modifying the original concentrations. Using gas-tight materials during PeriSIP seawater sampling, will allow to obtain samples, which neither loose nor take-up external gases from the atmosphere.

**Figure 4.24: Downstream seawater analyses with InEx samples collected with PeriSIP and VacuSIP.** Indicated are the maximum volumes ($V_{\text{max}}$), that have been either reported in literature for the VacuSIP or experimentally tested in the laboratory for the PeriSIP.

**EFFORT BY THE INVOLVED PERSONNEL**

This proposed PeriSIP setting is user-friendly, just requiring minimal manipulation during the deployment of the device, and minimal operations at the recovery after the desired sampling period. The complete pump system is small, light-weight, robust and easily transportable by a scuba-diver to the sampling site. Compared to the VacuSIP, no prior preparation of inducing vacuum in vials is required. The expandable urine bags used for collection do not float and are easily transportable under water. The procedure further ensures affordable costs. Costs for a pump production are estimated to be approximately 1000 euros per pump, making this technique economically affordable. Furthermore, the proposed procedure requires minimal material costs of consumables and little waste production. Other methods require many single components and the substitution of tubing material and/or syringes throughout the sampling procedure. The hereby used sampling tubes can be washed and reused, the only disposable material are the collecting bags. A major aim of this easy handling and affordable device is to further encourage InEx studies.
of filter-feeding organisms, promoting higher publication performance, and thus broaden our knowledge for the significance and mechanisms of InEx fluxes in marine ecosystems.

**HOT-BEAD-FLOW-SENSOR**

The flowmeter based on a Hot-Bead-Flow-Sensor will add significant advantages to future ‘PeriSIP’ devices. The flowmeter will measure automatically if excurrent jets are present, quantify the flux rates, note if target fluxes have the desired pre-defined flux speed and then trigger the pump rotation and water displacement to control sampling. This means that if a target animal stops pumping, or the manipulator is displaced by some external, undesired event, the pump will stop pumping and contamination by ambient water is avoided. The flowmeter will further allow to sample faster or slower depending on the flux rate and remain in a pre-defined optimal sampling range. It can further be applied in pilot studies to measure initial flux rates. Conclusively, the flowmeter adjusts sampling rates automatically, autonomously and in real-time depending on a present target flux. Future integration into the PeriSIP is planned and test results will be provided.

**LESSONS LEARNED FROM AQUARIUM AND IN SITU INEx SAMPLING OF C. RENIFORMIS**

**RENIFORMIS**

The application of the PeriSIP in aquarium conditions and in situ was applied to study InEx fluxes of the demosponge *Chondrosia reniformis*. During the pilot study we first revealed the sponge individual pumping rates in real time, and then sampled at 10% of the flux rate to obtain contamination free InEx samples. Natural InEx fluxes of *C. reniformis* retained planktonic cells at rates of > 97% for heterotrophic bacteria, >86% for *Synechococcus* spp., >95% for *Prochlorococcus* spp. and >70% picoeukaryotes. Although cell counts for *Synechococcus* (500x) and *Prochlorococcus* (325x) cells were distinctly lower, while heterotrophic bacterial levels were more than two-fold higher in the aquarium seawater, retention rates between aquarium and in situ conditions were very similar.

Our results are supported by prior works, which aimed at studying the feeding activity of *C. reniformis* on planktonic cells. *Synechococcus* cells are generally classified as preferred food source for sponges (Perea-Blázquez et al., 2012b; Topçu et al., 2010) and highest retention for *Synechococcus*-like cells were reported at 96% efficiency (Jiménez and Ribes, 2007; Nemoy et al., 2021). It has been also reported that sponges feed on picoplankton cells with almost 100% efficiency, ranging in cell sizes between 0.2 and 2 µm (Ribes et al., 1999; Topçu et al., 2010). Similar to our outcomes, Morganti et al. (2016) and Nemoy et al. (2021) reported a picoplankton retention efficiency of approximately 70% for *C. reniformis*. However, their results for *Prochlorococcus* cells
revealed lower retention efficiencies compared our findings – 7% Morganti et al. (2016) and 16% Nemoy et al. (2021); as did the retention for heterotrophic bacteria – 45% (Nemoy et al., 2021). The lower cell retention efficiencies reported by Nemoy et al. (2021) are likely related to their sampling method, other than a different feeding behavior in C. reniformis. They simply siphoned InEx samples through two 1 m long Teflon hoses with an internal diameter of 0.05 mm and glass capillary tips into test tubes (Figure 4.25). It is highly likely that their sampling strategy increased the risk of contaminating InEx samples with ambient water.

![Figure 4.25: InEx sampling method using a simple siphon mechanism (Nemoy et al., 2021).](image)

Net fluxes of inorganic nutrients showed consistent trends for InEx fluxes sampled from C. reniformis in situ and in aquarium conditions. In both conditions, C. reniformis acted as a sink for ammonium, while releasing nitrate back into the environment. This is consistent with current literature, that describes sponges as consumers of exogenous ammonium, while excreting nitrate as a metabolic outcome of microbe-mediated nitrification processes (Corredor et al., 1988; Diaz and Ward, 1997; Jiménez and Ribes, 2007; Nemoy et al., 2021; Ribes et al., 2012; Schläppy et al., 2010). Sponges produce endogenous ammonium as metabolic waste which, in nitrogen-limited environments, represents a profitable N source for associated microorganisms (Han et al., 2013). Microbial nitrification is performed to obtain energy from oxidizing the endogenous ammonium first to nitrite and then to nitrate (Kowalchuk and Stephen, 2001; Pita et al., 2018; Ribes et al., 2012; Schläppy et al., 2010), which in turn is released by the sponge with the exhaled seawater. Our results further confirm trends for DIN (dissolved inorganic nitrogen) consumption and release by C. reniformis, as proposed previously by Morganti et al. (2016) using ‘VacuSIP’.
Interestingly, ammonium consumption and nitrate release by *C. reniformis* increased in aquarium conditions, which was generally enriched in nitrogen compounds, including ammonium, nitrite, nitrate and total organic nitrogen. In accordance to previous findings of Jiménez and Ribes (2007), Bayer, Schmitt and Hentschel (2008), Schläppy *et al.* (2010) and Ribes *et al.* (2012), our results support that the microbe-mediated nitrification in the HMA sponge *C. reniformis* is enhanced under elevated ammonium concentrations in the water inhaled by the sponge. The recent study of Nemoy *et al.* (2021) examined the effect of ammonium enrichment on the microbe-mediated nitrification in *C. reniformis*. In laboratory experiments, they exposed sponge explants to ammonium enriched seawater (0.3 – 6.7 µM) and to ambient seawater (0.05 – 1.5 µM ammonium). In comparison, our mean ammonium concentrations in the aquarium and *in situ* were 3.4 µM and 1.0 µM, thus comparable to the tested concentration of their study. Despite observing ammonium uptake and nitrate release in InEx fluxes of *C. reniformis*, they could not show any relationship of increased inhaled ammonium to the consumption of ammonium, as well as the excretion of nitrate. In disagreement to our conclusions, Nemoy *et al.* (2021) suggest that the nitrification activity of sponge-associated microbes is not necessarily related to the concentration of ammonium in the surrounding seawater.

Organic carbon compounds, both total and dissolved organic carbon, were released at high concentrations in exhaled seawater during the *in situ* experiment. Surprisingly, in aquarium conditions the opposite trend was observed, where *C. reniformis* greatly retained organic carbon compounds. It can be speculated, that the release of organic carbon is only physiologically feasible at an optimal feeding-state of the sponge. Sponges can acquire carbon from a wide range of food sources in the water column, including DOC, but also larger forms such as particulate organic carbon, which includes living carbon such as heterotrophic bacteria, *Prochlorococcus* spp., and *Synechococcus* spp. (Ribes *et al.*, 1999). In our aquarium conditions, *Synechococcus* and *Prochlorococcus* cell counts were drastically reduced (500x and 325x, respectively), while heterotrophic bacteria concentrations were doubled. In this microplankton-poor environment, the sponge holobiont might have been experimenting a sort of ‘starving’ state, which led to no excess of organic compounds, finally resulting in a strong retention of particulate organic carbon.

**POSSIBLE LIMITATIONS OF THE ‘PERISIP’**

The ‘PeriSIP’ sampling method showed its versatile applicability in aquarium and *in situ* conditions and revealed high resolution for downstream analyses. However, possible limitations for the application *in situ* are geophysical factors of target reef systems and ocean currents. To sample InEx fluxes, it is necessary a stable installation of custom-built manipulators, which hold the sampling tube in an exhalant orifice such as an osculum. Cavities in uneven rocks represent suitable anchorages for the deployment of the device and manipulator. However, the sampling of InEx fluxes...
from filter-feeders living on flat vertical walls is more challenging, and requires ability to install manipulators with screws and/or epoxy resin, or the use of larger manipulators deployed on the sediment and reaching the target animal.

One critical step during the installation is the correct positioning of the sampling tubes inside of exhalant openings, such as the oscula, without touching the animal. This requires prior training of a scuba diver in aquarium conditions and practical testing in situ prior to sampling. The difficulty to insert the sampling tubes inside of the filter-feeder is increasingly difficult under strong currents, which further increase the risk of manipulator displacement and require addition of weights or fastening systems to ensure stability.

**POSSIBLE SCIENTIFIC AND INDUSTRIAL APPLICATIONS OF THE ‘PeriSIP’**

The ‘PeriSIP’ device was developed for marine biologists which are interested in studying the functional role of InEx fluxes from filter-feeders. By introducing an automated underwater peristaltic pump system, the PeriSIP provides a novel, non-intrusive in situ method largely improving the effectiveness and sample quality of existing state-of-the-art methods. Downstream analyses of InEx samples may cover a wide range of scientific question by analyzing a) particulate organic matter (POM), including planktonic and microbial cells and the quantification of their removal efficiencies b) filtration and feeding rates of dissolved inorganic and organic nutrients, c) nutrition and metabolism (in terms of biomolecules transformations) d) respiration activities, such as gas transformations and e) chemical compounds and metabolites present in InEx fluxes of active suspension feeders.

In innovative approaches for climate change management, a potential applicability of PeriSIP could be to study whether filter-feeders activity contributes significantly to neutralize greenhouse-gas concentrations such as CO₂, N₂O and CH₄. The Federal Ministry of Education and Research in Germany for example, are sponsoring initiations like sea4socIety (‘SEA4SOCIETY’) to search solutions for carbon-sequestration in coastal ecosystems. Related projects are interested in quantifying the storage capacity of “blue carbon” to estimate the potential in mitigating climate change.

In the field of bioremediation, the PeriSIP could be of great use in assessing and monitoring filter-feeders as biofilters used in aquaculture and disease management. Filtration, e.g. by bivalves, can augment or reduce disease outcomes via removal of pathogens from the water column, or the release of pathogens in pseudofeces, respectively. The effect of filtration on pathogen transmission depends on the selectivity of the filter-feeder and needs prior evaluation and monitoring for secure applications. Furthermore, climate change may affect the potential for filter-feeders to mitigate disease risks. Less studied invertebrates like ascidians and sponges need scientific evaluation to estimate how efficiently they might contribute to ecosystem services by altering pathogen
transmission (Burge et al., 2016; Cranford et al., 2011). The proposed InEx sampling device and downstream procedures can inform accurately about the actual activities of the involved filter feeding assemblages. In this sense, we can gather information about what is present in the water column, and what these organisms are filtering, eliminating, metabolizing and releasing to the system.

The PeriSIP can also be perfectly applied to non-living fluxes of water, requiring precise coupling or positioning with respect to a localized fluid or gas flux. Researchers which aim to collect and study marine porewater profiles, such as present in seagrass-vegetated sediments, can profit from this sampling technique.

**CONCLUSION**

The ‘PeriSIP’ allows to sample InEx water fluxes of filter-feeding organisms *in situ* in a precise manner, with an efficient, non-intrusive approach and without negative impacts on the organism or the environment. As a light-weight and small sampling device, the ‘PeriSIP’ is interactive and usable by any research diver under water. The procedure offers a big spatial resolution to collect water samples from specific, defined points of a directed flux. In particular, large volumes of InEx fluxes can be collected from filter-feeders with highly minimized risks of contamination, giving the chance to perform a wide array of downstream analyses to reconstruct a complete picture of the interaction between the organisms and their environment. Until now, collecting large volumes of clean InEx fluxes from filter feeding communities has been a major humper. The ‘PeriSIP’ method transcends the boundaries of current research in the topic in multiple directions by: a) precisely collecting larger volumes of clean InEx fluxes as a standardized method; b) being applicable for multiple flux studies from a wide array of filter-feeding species; c) providing a user-friendly and low-cost device highly versatile to perform field *in situ* and laboratory aquaria studies; d) minimizing waste material and hand effort in its usage; and e) enhancing mechanistic understanding of the functional roles of filter-feeders in the marine environment, minimizing confounding effects resulting from unprecise sampling methods. The ecological interactions of sponge holobionts and larger Porifera assemblages with their environment are essential aspects to reconstruct ecosystems functioning in future studies. In particular, microbial and biochemical exchanges and retention, and DOM, POM and metabolite fates and transformations, either by host cell or associated microbes are key elements to be studied utilizing PeriSIP sampling, in combination with downstream approaches mentioned in the previous sections.


CONCLUDING REMARKS

This PhD thesis adopts an integrative, multidisciplinary approach to study the acclimatization of two Mediterranean marine sponges – the high-microbial abundance (HMA) sponge *Chondrosia reniformis* and the low microbial abundance (LMA) sponge *Spirastrella cunctatrix* – to ocean acidification (OA). These sponges inhabit a volcanic cold seep system off Ischia Island (Italy), and this study employs *in situ* observations, Next-Generation-Sequencing metabarcoding, and NMR-based metabolomics to investigate, for the first time, the impact of OA on these long-term adapted sponge species. This study provides the first documentation of NMR spectra for these two sponge species, as well as the first report of differential abundance of microbes with associations to both vent and control sites. The results of this investigation provide novel insights into the microbial ecology of these sponge species and their associated environments. Phenotypic adjustments in host morphology, microbial community composition, and metabolite profiling are uncovered, providing new insights into the adaptive traits under OA. This thesis contributes to current knowledge by emphasizing that both LMA and HMA sponges can acclimatize to OA and undergo microbial restructuring, such as symbiont switching and symbiont shuffling. While sponge hosts benefit from a certain degree of microbiome dynamics, maintaining a stable metabolome appears to correlate with healthy sponge morphology. Conversely, signs of microbial dysbiosis and significant metabolome changes seem to correlate with impaired sponge morphology, such as the reduction of body size and aquiferous systems (see Figure 3).

Figure 3: Metabolic and microbial adaptive traits to OA in the HMA and LMA species, *C. reniformis* and *S. cunctatrix*, respectively. In the HMA species, no morphological changes, as well as no significant metabolome changes were observed. The microbial community changed significantly in beta diversity, decreased core taxa and showed no signs for dysbiosis, reflected in decreased alpha-diversities and constant beta dispersion. In
contrast, the LMA sponge showed in vent conditions significantly higher levels of metabolites such as betaine, amino acids, lactate, lipids and choline. Significantly decreased metabolite levels were observed for taurine, trigonelline, creatine, allantoin and AMP. Together with significant microbiome changes, such as beta diversity and increased number of core taxa, three signs for microbial dysbiosis were observed, manifested in increased alpha diversity, beta-diversity, seawater taxa and pathogenic strains. These metabolic and microbial changes are correlated with morphological impairment, such as a reduced aquiferous system and body surface at the CO₂ vent site.

**THE HMA SPECIES - CHONDROSIA RENIFORMIS**

The HMA sponge *C. reniformis* displayed no morphological impairment and was highly abundant at the CO₂ vent site, suggesting that *C. reniformis* holobionts are well-adapted or resilient species. Although the microbial community changed across sites, mostly microbes with putatively similar functions were maintained, exhibiting microbial-metabolite functional redundancy. The microbial community displayed changes in beta diversity metrics, including the acquisition and depletion of ASVs (symbiont switching), and changes in the relative abundances of microbial taxa (symbiont shuffling). The overall number of core taxa and alpha-diversity decreased. Several microbial taxa, including *Endozoicomonas*, *Dadabacteriales*, *Bdellovibrio*, and *Candidatus Kaiserbacteria*, were differentially more abundant at the vent site. Within-community variabilities (beta dispersion) across sites did not change significantly, suggesting that the HMA holobiont reshaped its microbiome as a positive adaptation, rather than a bypass stress arrangement. It is possible that another microbial phenotype of *C. reniformis* developed over generations at the vent site, maintaining key functions and organismal health. This was further reflected in the metabolite profiles of the HMA sponge, which exhibited similar equivalent patterns across sites. These results increase our understanding of adaptive patterns to OA and contradict existing aquarium experiments that suggested no microbial restructuring of the *C. reniformis* microbiome (Ribes et al., 2016). It can be concluded that *C. reniformis* was able to maintain sturdy stability under different pH conditions on various biological levels, including morphology, microbiome, and its metabolome.

**THE LMA SPECIES - SPIRASTRELLA CUNCTATRIX**

*In situ* assemblages of the LMA sponge *S. cunctatrix* differed notably across the control and the vent site, showing signs of morphological impairment through a distinctly lower body surface area, smaller water canals and oscula, paler coloration and overall signs of tissue necrosis. Microbiome results suggested signs for microbial dysbiosis and instability, such as increases in alpha diversity (likely including opportunistic strains), larger intragroup dispersion and a shift from sponge enriched microbes towards seawater microbes. Vent associated differentially abundant microbes included *Zeaxanthinibacter*, *Nitrosococcales* (AqS1), *Nitrospira* and *Thermoanabaculaceae*. Several shifts with functional correlation between differentially abundant microbes and relative metabolite shifts were detected. At the vent site, taurine levels decreased, accompanied by
reduced differential abundance of the taurine utilizing core symbiont UBA10353 (Baltar et al., 2022). Decreased taurine levels, probably reduced the reproductive kinetics of the gammaproteobacterial UBA10353, finally resulting in decreased microbial abundances due to out-competition by other microbial taxa. Consequently, beneficial functions performed by this symbiont were possibly diminished, including general carbon and sulfur metabolism via chemoautotrophic carbon fixation and thiosulfate oxidation (Burgsdorf et al., 2022), as well as the production of secondary metabolites (mycalamide) with antiviral and apoptotic effects (Hood et al., 2001). Increased arginine concentrations at the vent site were reported along with higher abundances of the symbiont AqS1. This symbiont was found to be highly abundant in the inner cell mass of *Amphimedon queenslandica* larvae, where it synthesizes arginine from citrulline and potentially supplies larval cells with this amino acid (Song et al., 2021). Arginine is further metabolized by the nitric oxide synthase, to produce nitric oxide. Given that nitric oxide facilitates larval settlement and regulates metamorphosis in the sponge *Amphimedon queenslandica* and in diverse other marine phyla, including molluscs, ascidian, bryozoan, sea urchins and gastropods (Bishop and Brandhorst, 2007, 2003; Castellano et al., 2014; Leise et al., 2001; Pechenik et al., 2007; Ueda et al., 2016; Ueda and Degnan, 2013; Yang et al., 2018), it could be hypothesized that this physiological mechanism supports reproduction and survival in *S. cunctatrix* at increased seawater acidification.

The incidence of tissue necrosis exhibited at the vent site, could be related to a combined effect of increased presence of pathogenic Flavobacteriales strains and decreased allantoin concentrations. Allantoin promotes tissue growth and wound repair (Wishart et al., 2022), whereas some Flavobacteriales species were found to induce severe skin lesions and necrosis in fish via degradation of complex acidic polysaccharides of connective tissue (Declercq et al., 2013). Mechanistic insights into the proposed pathways could be target of future studies, using quantitative PCR to measure expressed mRNA levels of responsible genes, or transcriptomics for a more global view of gene transcripts.

**PERI SIP – DEVELOPMENT OF AN EFFICIENT SAMPLING METHOD OF INEX FLUXES FROM FILTER-FEEDING ORGANISMS FOR THE STUDY OF LOCAL SEAWATER CHANGES UNDER OA**

The novel PeriSIP methodology presented in this thesis, aims at collecting precisely inhaled and exhaled (InEx) water fluxes from filter-feeders, using constant sampling rates and allowing the collection of large fluid volumes. Mainly due to its increased sampling efficiency and quality, this novel sampling device presents a strong improvement over existing methods and advances current
scientific methods in filter-feeder research. One of the biggest advantages is that sampling rates can be adjusted depending on the studied target water fluxes. This opens up the possibility to sample from different organisms such as ascidians, bivalves and diverse sponge species, which naturally have different filter-feeding velocities. The sampling procedure works autonomously for up to 8 hours after correct in situ installation, allowing to collect large sample volumes and perform several downstream analyses and study designs. This allows to obtain deeper insights into key ecological mechanisms changing the microbiology and biochemistry in aquatic system communities (e.g., in control versus CO₂ vent sites with different pH).

**Future Directions**

This study aimed to shed light on the microbial and metabolic dynamics of two Mediterranean sponge species in response to OA. Microbiome results suggest that both the HMA sponge *C. reniformis* and the LMA sponge *S. cunctatrix* restructured their microbiomes via changes in relative abundances and differentially abundant microbes of symbionts (symbiont shuffling), as well as the acquisition or depletion of new ASVs in the vent site (symbiont switching). Both sponge species showed different core microbiomes across sites, suggesting an adaptive re-composition of functionally important core microbes under OA. The capacity for these microbial restructuring mechanisms should be further confirmed in more HMA and LMA sponge species and different control sites and vent systems to identify generalizable versus species-specific trends in microbiome dynamics and core microbiome adaptation under OA. It would be worthwhile in upcoming studies to estimate the weight of horizontally versus vertically transmitted microbes that take part in the core microbiomes, and under control and OA scenarios. This could be empirically approached by analyzing incurrent and excurrent filtering fluxes and larval stages, using metabarcoding and FISH (Fluorescent in situ Hybridization) techniques (Moter and Göbel, 2000). Furthermore, to support microbial flexibility as an adaptive mechanism within one sponge generation, long-term reciprocal transplant experiments could be performed whereby sponge individuals are introduced from control sites into CO₂ vent sites and vice versa. A community shift from control microbiomes towards vent microbiomes and vice versa would support microbial flexibility as a key mechanism of host survival under OA (Kandler et al., 2018).

The direct correlation between microbial taxa and their functional role within sponge holobionts needs further investigation. The metabolic functions discussed here, which are putatively performed by sponge-associated microbiomes, are estimates and speculative interpretations based on genetic information of cultivated microbial taxa or single-cell genomic analyses. Further validation will be required to improve mechanistic understanding of holobiont components. Analysing whole genomes will allow the identification of the general microbial gene repertoire and
putative functions. Genetic and metabolic pathways that become up and down regulated in host cells and the microbial compartment in sponges from control sites and vent systems can be assessed with meta-transcriptomics studies. These could also reflect the expression levels of certain immune response genes that affect microbiome structure and host-microbe interactions (Posadas et al., 2022). The investigation of transcripts encoding essential proteins that participate in nutritional and biochemical cycling, including carbon, nitrogen, sulfur, phosphorus, and vitamin B1 cycling, is crucial for comprehending the changes in microbiome and host functionality under OA stress. Furthermore, it is widely assumed that symbiotic microorganisms exchange molecules such as nutrients, vitamins, and allelochemicals with their hosts, but this is still unstudied in acidified systems. Hence, stable isotope signatures and other isotopic techniques will be crucial to test these trophic biochemical relationships under OA conditions and better understand host-microbe interactions.

This doctorate study provided the first NMR spectra for C. reniformis and S. cunctatrix and identified possible NMR variable candidates as discriminant metabolites across sampling sites. Revealed metabolic trends were based on OPLS-DA, a multivariate prediction modelling, and need to be complemented in future studies, as models are predictions with variable predictive accuracies. Comparing sponge metabolic patterns between vent and control sites, and quantifying key metabolites that might be involved in acclimatization processes via untargeted and targeted metabolomics profiling will be of key significance. Although, the identification of metabolites through NMR is both reproducible and distinctive, incorporating MS spectrometry in conjunction with NMR would enhance the accuracy of metabolite identification, as noted by Pan and Raftery (2007). Additionally, this thesis exclusively examined NMR variables that have been assigned, and it is highly probable that the natural complexity of sponge responses to OA encompasses numerous other compounds that have yet to be discovered. It would be intriguing to investigate the degree of impact on homeostatic primary versus secondary metabolism in acidic conditions by analysing complete NMR and LC-MS spectra. An important area for future research would be to investigate whether potential biochemical changes arising from OA are primarily driven by variations in the host cells or alterations in the microbiome. Such hypotheses could be addressed with stable isotope analysis on separate compartments of host and symbiont cells for total C, N, or other atom-type compound contributions, pulse-chase experiments, and specific marked compound approaches.

In forthcoming investigations, it is essential not only to elucidate the mechanisms underlying sponge acclimatization and the associations between host or microbial partners and altered metabolic pathways under OA, but also to describe the role of Porifera holobionts in the biochemistry of their surrounding environments. This can be accomplished by analysing their biochemical turnover resulting from their filter-feeding activities and comparing inhaled and exhaled seawater profiles for nutrients and metabolites. The PeriSIP technology presented in this
thesis can be applied to whole filter-feeding assemblages in CO₂ vent and control sites. This will enable the estimation of how key ecological processes, including nutrient cycling and trophic coupling, might be affected under OA. The analysis of inhaled and exhaled seawater samples downstream may encompass a wide range of scientific questions. These questions may include, but are not limited to: a) quantifying the removal efficiencies of particulate organic matter (POM), such as planktonic and microbial cells, b) determining filtration and feeding rates of dissolved inorganic and organic nutrients, c) investigating nutrition and metabolism (in terms of biomolecule transformations), d) assessing respiration activities, such as gas transformations, and e) identifying chemical compounds and metabolites present in InEx fluxes of active suspension feeders. The examination of ecological interactions between sponge holobionts, larger Porifera assemblages, and their environment is crucial for reconstructing ecosystem functioning and making more accurate predictions regarding the future of our oceans.

CLOSING REMARKS

The variety of species-specific responses on microbial, physiological, morphological, and biochemical pathways indicates that not all sponge species can be classified as winner or loser taxa in future climate change scenarios. The HMA and LMA status appears to play a significant role in organismal adaptation trends, but species-specificity remains decisive. Microbiome rearrangements in the form of positive adaptations appear to be beneficial for sponge holobionts in a fluctuant environment, provided that metabolic homeostasis is maintained. Symbiotic interactions modulate organismal physiologies and influence the surrounding environment, acting at the species level and up to the functional level of ecosystems. Overall, symbiosis continues to demonstrate itself as the key evolutionary engine in species acclimatization, adaptation, and resilience for both present and future oceans.

This PhD project allowed me to develop several skills in scientific data analysis, such as the bioinformatic analyses of Illumina sequencing data, chemical structure elucidation of NMR spectral data, and diverse statistical analyses in R studio. During the theoretical design and practical realization of the new submarine sampling device ‘PeriSIP’, I was able to coordinate international collaboration with the Max-Planck-Institute in Bremen (Germany) and visited the research facility, which greatly increased my scientific networking experience. I was also able to improve my diving skills during the underwater work, including scuba and free diving. Presenting first results at an international conference, the 32nd ESCPB, allowed me to further enhance my presentation and communication skills. In conclusion, this PhD work allowed me to grow personally and as a researcher.
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