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ABSTRACT

The EU Animal By-Products Regulations generated the need for novel methods of storage and disposal of dead livestock. Bioreduction prior to rendering or incineration has been proposed as a practical and potentially cost-effective method; however, its biosecurity characteristics need to be elucidated. To address this, *Salmonella enterica* (serovars Senftenberg and Poona), *Enterococcus faecalis*, *Campylobacter jejuni*, *Campylobacter coli* and a *lux*-marked strain of *Escherichia coli* O157 were inoculated into laboratory-scale bioreduction vessels containing sheep carcass constituents. Numbers of all pathogens and the metabolic activity of *E. coli* O157 decreased significantly within the liquor waste over time, and only *E. faecalis* remained detectable after three months. Only very low numbers of *Salmonella* spp. and *E. faecalis* were detected in bioaerosols, and only at initial stages of the trial. These results further indicate that bioreduction represents a suitable method of storing and reducing the volume of livestock carcasses prior to ultimate disposal.

*Keywords***:** agriculture; animal slaughterhouse waste; fallen livestock; meat waste; zoonoses

1. Introduction

In order to reduce the risk of further outbreaks of animal diseases such as bovine spongiform encephalopathy and foot and mouth disease, the European Union introduced the Animal By-Products Regulations (EC/1774/2002) in 2003 (Anon, 2009). These regulations sought to improve biosecurity across all aspects of the livestock sector, from production to waste disposal. Since their implementation, the options available to most farmers to dispose of fallen (dead) livestock have been effectively limited to either rendering or incineration, whereas previously most fallen stock was buried. The regulations have led to animosity within the agricultural industry due to the considerable costs and biosecurity concerns associated with centralised collection and rendering or incineration of fallen stock (Bansback, 2006; Gwyther et al., 2011). Indeed, there is call for both a change in legislation and the development of alternative methods of disposal (Bansback, 2006).

Bioreduction is a novel technology that has shown potential as a viable option for storing and pre-treating fallen stock prior to disposal (Williams et al., 2009). Bioreduction is the aerobic biodegradation of animal by-products in a partially sealed vessel, where the contents are mildly heated and aerated and ultimately disposed of via the permitted route for 'Category 1' material in accordance to the EU ABPR (i.e. via incineration or rendering). The process has been shown to reduce the volume of waste and hence the frequency of collection and associated disposal cost, as well as being a practical method for industry (Williams et al., 2009).

Dead livestock may harbour a range of zoonotic agents (Milnes et al., 2008), and current methodologies for their disposal in Europe (e.g. incineration and rendering)

depend on high temperatures to deactivate pathogens; however, bioreduction operates at a mesophilic temperature (approx. 40 °C) and does not utilise any chemical disinfection procedure. Rather, the active aeration coupled with the competitive and antagonistic effects of the prevalent microbes are hypothesised to reduce pathogen levels (Williams et al., 2009). For bioreduction to be approved under the revised EU ABPR (EC/1069/2009) (as described in Annex IV of EU implementing Regulation EC 142/2011) as an alternative method of storing fallen stock prior to disposal, the fate of pathogens within the system must be elucidated and the evidence presented to the European Food Safety Authority (EFSA), which then decide whether to ratify the system for industry use (Bohm, 2008). EFSA stipulate that novel disposal methods should lead to a 5-log reduction in the numbers of two indicator organisms representing bacterial pathogens, *Salmonella enterica* serovar Senftenberg (hereafter called *S.* Senftenberg) and *Enterococcus faecalis* (Bohm, 2008). A previous field-scale study on bioreduction of sheep recovered negligible numbers of pathogens (Williams et al., 2009), but the initial pathogen concentration was not high enough to validate whether or not a 5-log reduction in numbers had occurred. Whilst it is preferable to assess the fate of pathogens at field-scale, the logistics of growing and handling the large volumes of pathogens needed to gain a sufficient concentration in the bioreduction vessels would be problematic. Further, EFSA guidelines state that simulated systems can be used as a proxy of field-scale systems provided that they are representative of actual conditions (EFSA, 2008).

The aim of this work was to validate the effectiveness of bioreduction in reducing numbers of introduced pathogens in a laboratory-scale system. By applying the criteria stipulated by EFSA for ratifying novel *disposal* methods to a simulated *storage* process

that is bioreduction, this study will help verify whether bioreduction represents a biosecure method of containing fallen stock prior to disposal. In addition to *S.* Senftenberg and *Enterococcus faecalis*, additional microorganisms (*Campylobacter* spp., *E. coli* O157, and other *Salmonella* strains) were also tested as they represent common zoonotic pathogens that may be introduced with carcasses into bioreduction vessels.

2. Materials and methods

2.1 Vessel design

Laboratory-scale versions of the bioreduction vessels described by Williams et al. (2009) were constructed using 5 l polypropylene containers; 19 cm high × 13 cm wide × 26 cm long. These mini bioreducer vessels (MBVs) were placed within a darkened incubator set to 40 °C (± 2 °C) and the contents continuously aerated at a maximum rate of 6 l min^{-1}. To negate microbial contamination and odour, the outflow from the MBVs were passed through a commercial disinfectant (20% Trigene; Medichem, Kent, UK) and then an odour trap containing activated charcoal before being vented into a Category II biosafety cabinet (Fig. 1).

2.2 Trial management

The inoculated MBVs (n = 3) were managed in a similar way to the field-scale bioreduction vessels (Williams et al., 2009). Specifically, an initial volume of water (2.2 l; equivalent to 2,800 l under field-scale conditions) was added so that each MBV was just under half filled. A total of 231 ± 1.5 g of sheep carcass components (equivalent to

300 kg under field-scale conditions) were added to each MBV. Components (sourced locally) comprised of muscle, bone, fat (in an intact 'chop'), pelt, blood, stomach contents, wool and liver, in proportions representative of a sheep carcass (MLC, 2006), to give a final volume of approximately 2.5 l, determined by volume marks on each MBV and by weight. Particle size was uniform across all MBVs, up to a maximum of approximately 100 mm (bone), with components being distributed uniformly within each MBV. A commercial catalyst with sodium alginate as the active ingredient (Gel-60®, Biopolym, Spain) was added at the recommended dose of 1 g catalyst to 1 kg of carcass (Williams et al., 2009). The final ratio of liquid to solids was 10:1. Each MBV was aerated as described previously. Water levels were visually inspected every other day and fresh water added so that two-thirds of the carcass contents (by height) were covered throughout the trial (Gutiérrez et al., 2003; Lobera et al., 2007). When individual carcass components were no longer recognisable, water was added to prevent drying out of the remaining organic matter. Control MBVs (n = 2) were managed under the same conditions but without the addition of pathogens.

2.3 Inoculation

2.3.1 Bacterial strains

S. Senftenberg (NCTC13385) was obtained from HPA culture collections (Health Protection Agency, Salisbury, UK) and *Enterococcus faecalis* (ATCC 29212) was obtained from Oxoid (Oxoid Ltd., Hampshire, UK). *Salmonella enterica* (ser) Poona (hereafter called *S.* Poona) (NCTC4840) was obtained from Oxoid; a *lux*-marked strain of *E. coli* O157 (3704 Tn*5 luxCDABE*) and an environmental strain of *E. coli* O157 (#3704)

(both non-pathogenic but accurately reflecting survival of pathogenic strains) were used to represent verocytotoxic *E. coli* (Kudva et al., 1998; Ritchie et al., 2003); and *Campylobacter jejuni* (6035) and *Campylobacter coli* (6168) were kindly donated by the University of Aberdeen (Ogden et al., 2009). All media were bought from Oxoid unless otherwise stated.

2.3.2 Microbiological preparation and inoculation

All bacterial strains were grown from frozen stock, with each (except *Campylobacter*) grown overnight in tryptone soya broth (TSB; CM0129) then subsequently combined with other strains of the same organism and incubated overnight in fresh TSB in an orbital shaker (150 rev min^{-1}) at 37 °C. *C. coli and C. jejuni* were grown overnight in Bolton Broth (CM0983) containing lysed horse blood (SR0048) and a supplement containing cefoperazone, vancomycin, trimethoprim and cycloheximide (SR0183) in a microaerobic environment at 41.5 °C and subsequently combined and again incubated overnight. The microaerobic environments were obtained using anaerobic jars and CampyGen sachets (CN0025).

The final concentration of each inoculum was obtained by serially diluting and plating onto selective agar: *Salmonella* on Xylose-Lysine-Desoxycholate Agar (XLD, CM0469); *E. coli* O157 on Sorbitol MacConkey Agar (CM0813) containing cefixime and potassium tellurite (CT-SMAC; SR0172); *E. faecalis* on Slanetz and Bartley Medium (SBM; CM0377) and *Campylobacter* on modified Charcoal Cefoperazone Deoxycholate agar (mCCDA; CM0739) containing the supplement cefoperazone and amphotericin B (SR0155). SBM plates were incubated for 48 hours at 37 °C whereas mCCDA plates

were incubated microaerobically for 48 hours at 41.5 °C. All other plates were incubated at 37 °C for 24 hours. Each treatment MBV was subsequently inoculated so that the concentration of micro-organisms per ml of liquid was as follows: 7.91 $\log_{10}$ CFU of *Salmonella*, 7.89 $\log_{10}$ CFU of *E. faecalis*, 7.5 $\log_{10}$ CFU of *E. coli* O157 and 6.81 $\log_{10}$ CFU of *Campylobacter*.

2.4 Liquor waste

2.4.1 Microbiological analysis

2.4.1.1 Enumeration

Liquor samples (25 ml) were recovered directly from each MBV using a 5 ml pipette on days 0, 3, 23, 56 and 84 and analysed on the same day. Samples were homogenised in a Seward 400 stomacher (Seward Ltd., Worthing, UK) for 1 min at 230 rev min^{-1} with 225 ml maximum recovery diluent (MRD, CM0733) then serially diluted in MRD. Samples were enumerated as described previously for each pathogen. Total viable counts (TVC) were enumerated using the pour plate techniques based on and BS EN ISO 4833:2003 (FSA, 2009a) using standard plate count agar (PCA, CM0463). All presumptive colonies were sub-cultured onto nutrient agar and incubated at 37 °C for 24 hours, whilst presumptive *Campylobacter* colonies were incubated microaerobically at 41.5 °C for 48 hours. *E. coli* O157, *Campylobacter* and *Salmonella* spp. were confirmed using latex agglutination (DR0620, DR0150 and FT0203, respectively) with further biochemical tests using Microbact™ GNB 12A (MB1132) and sub-culturing onto *Salmonella* chromogenic agar (CM1007 containing supplement SR0194) for *Salmonella*

spp.. Confirmation of *E. faecalis* was performed using glucose agar (Sigma, 16447) and subsequently sub-culturing onto bile aesculin agar (CM0888).

2.4.1.2 Enrichment

Where *Salmonella, E. coli* O157 and *Campylobacter* were not detected by enumeration, enrichment was used to confirm the absence of these bacteria. Enrichment of samples for *Salmonella* spp. was based on ISO standards 6579:2002 (FSA, 2009b) and *Campylobacter* samples were enriched based on the ISO 10272-1:2006(E) method (HPA, 2007). For the enrichment of *E. coli* O157, 20 ml of mTSB (CM0989 containing supplement SR0190) was added to 5 ml of liquor and shaken in an orbital shaker for 6 hours (37 °C, 150 rev min^{-1}) after which time 0.1 ml of the enriched culture was streaked onto duplicate plates of CT-SMAC. Plates were incubated and presumptive colonies confirmed as described previously.

2.4.2 Activity of E. coli *O157*

At each sampling time-point, a 1 ml aliquot from each MB was placed into a plastic luminometer cuvette and its luminescence [relative light units (RLU)] determined using a SystemSURE 18172 luminometer (Hygiena Int., Watford, UK).

2.5 Bioaerosol analysis

Bioaerosol samples were taken on days 0, 24, 57, and 85. Samples were obtained from gases before they passed through the commercial disinfectant using selective agar

plates in an Andersen Air Sampler 2000 (Andersen Instruments Inc; Atlanta, Georgia, USA). The pump was connected to the mini bioreducer by silicone tubing and activated for 30 min at a flow rate of 10 l min^{-1}. Plates were arranged randomly with each sampling date but were consistent between replicates on the same sampling date. *E. faecalis* were captured using Slanetz and Bartley Medium, *Salmonella* using Brilliant Green Agar (BGA, CM0263), *Campylobacter* using mCCDA, *E. coli* O157 using CT-SMAC, and TVC using PCA. All plates were incubated as described previously whilst the BGA was incubated at 37 °C for 24 h.

2.6 Data Analysis

Samples where micro-organisms were detected only via enrichment were allocated an arbitrary value of half the detection limit. To avoid analysing data with zero values (i.e. those not detected by enrichment), all data were $\log_{10}(y+1)$ transformed. Data were analysed using SPSS v15.0 (SPSS Inc., Chicago, USA). Normality of the microbiological data was tested using the Kolmogorov-Smirnov test and means analysed using either related samples *t*-test if normal or Wilcoxon signed rank test if non-normal.

3. Results

3.1 Waste degradation

At the end of the trial, the reduction in volume of carcass components in each vessel was similar (88.2 ± 3.7% of that initially added). The discernable animal remains

were predominantly identified as stomach content although there were also some fatty deposits and small fragments of bone.

3.2 Microbiological characteristics

The controls were found to have natural populations of *Salmonella* spp., *E. faecalis*, and *Campylobacter* spp. but no *E. coli* O157 were detected. Survival of the introduced *Salmonella* spp. and *E. faecalis* in the treatment MBVs followed similar survival patterns to natural populations in the controls (Fig. 2). Although numbers of both *Salmonella* spp. and *E. faecalis* reduced markedly over the three month trial, the dynamics of survival differed between both micro-organisms. Specifically, *Salmonella* spp. numbers remained relatively stable until day 54, after which they significantly declined (P <0.05) so that they could only be detected by enrichment at the end of the trial period (Fig. 2A). Numbers of *E. faecalis* generally decreased more steadily throughout the trial, although had recovered somewhat in the control MBs towards the latter stages (Fig. 2B) (P >0.05).

A significant (P <0.05) decline in both the numbers and activity of *E. coli* O157 were seen in the inoculated MBVs (Fig. 3A), culminating in a 5-log reduction by day 84 and luminescence values falling to below background levels. *Campylobacter* spp. numbers declined significantly (P <0.05) within the first three days followed by an increase in numbers in both the control and inoculated MBVs on day 23 and a subsequent decrease (Fig. 3B). On day 56, *Campylobacter* spp. numbers in the inoculated vessels had reduced by greater than 5-log values and remained so until the end of the trial. TVC values dropped initially in the inoculated MBVs, but then recovered and stabilised towards latter stages of the trial (Fig. 4). In the control MBVs, there was a non-significant

increase in numbers up to day 23 (P >0.05), followed by a gradual decrease to values very similar to those in the treatment bioreducers.

3.3 Bioaerosols

No pathogens were recovered as bioaerosols from the control bioreducers. Low numbers of *Salmonella* spp. and *E. faecalis* were detected as bioaerosols in initial stages of the trial from the inoculated MBVs; although no *Salmonella* were detected after the first sampling date and numbers of *E. faecalis* decreased considerably with each sampling date until they were undetectable (Table 1). Although this mimicked the decline in mean concentration of *E. faecalis* within the liquor, the relationship between bioaerosol and liquor counts was not statistically significant (P >0.05; data not shown). Neither *E. coli* O157 nor *Campylobacter* were detected within any bioaerosol samples. TVC values increased towards the middle and latter stages of the trial in both the inoculated and control systems and were statistically similar throughout (P >0.05); although numbers recovered were more variable with time.

4. Discussion

This trial was conducted over three months as it has been shown that this is the time required for most of the carcass components to degrade within a bioreduction system. Bioreduction has already proved to be effective at reducing the volume of carcass material to be disposed (Williams et al., 2009) and the findings of this trial supported this as even the bone material largely degraded. Although the system was designed to

accurately mimic field-scale bioreduction, it should be remembered that the surface area of the contents added to the MBVs was proportionately greater than that of an entire carcass and that the aeration rate was greater; caution is therefore needed when extrapolating the rates of degradation to field-scale.

In keeping with previous work on bioreduction (Gutiérrez et al., 2003, Williams et al., 2009), a catalyst product, Gel-60® (Biopolym, Spain), was used in this trial. Gel-60® is based on sodium alginate and it is claimed that the hydrolysis of fibre, proteins, poly- and oligo-saccharides within it provides ready substrates for the indigenous microbial community, encouraging microbial growth and degradation of the animal carcass (Gutiérrez et al., 2003). However, the efficacy of Gel-60® has recently been trialled and was found to induce no affect on degradation rates (data not shown).

At the end of the bioreduction process, there remains a nutrient-rich liquid that should be disposed of via the permitted route for 'Category 1' material according to the EU ABPR (Williams et al., 2009). Lobera et al. (2007) recommend retaining a proportion of the liquor to facilitate the bioreduction of future carcasses, as is advised to inoculate new compost or anaerobic digestion plants (Gerardi, 2003; Sundberg and Johnson, 2005). In addition to reducing the volume for final disposal, the advantage of liquid waste compared to solid is that it can be removed easily from the vessel by use of a vacuum pump. Relevant biosecurity and environmental factors to consider with bioreduction and the liquor waste generated have been explored and compared to other methods of carcass storage and disposal by Gwyther et al. (2011).

EFSA stipulate that novel disposal methods for animal by-products should lead to a 5-log reduction in the numbers of *S.* Senftenberg and *E. faecalis* as a measure of

biosecurity (Bohm, 2008). Bioreduction isn't however a disposal method for fallen stock, but rather a storage system designed to reduce the volume of waste in a safe, contained environment prior to ultimate disposal via incineration or rendering (Williams et al., 2009). Over the three month trial period, all microorganisms, with the exception of *E. faecalis*, had reduced by 5-log values, although *E. faecalis* had also notably decreased by over 4-log values. However, due to the low numbers of *Salmonella* spp. identified on the final sampling date, it is recommended that future trials go beyond the three month period in order to gain a better insight into the fate of the organism. A carcass storage system similar to bioreduction, though anaerobic, was trialled by Gutiérrez et al. (2003) and a number of commensal pathogens, including *Salmonella* spp., could not be detected after 55 days. Although their results concur with the ones obtained in this current study, a direct comparison isn't possible due to the difference in aeration status of the systems trialled and because Gutiérrez et al. (2003) looked at presence or absence of pathogens, rather than numbers. EFSA also stipulate that novel disposal methods for animal by-products should lead to a 3-log reduction in the numbers of suitable indicator viruses (Bohm, 2008). Viruses were not analysed in this trial due to logistical issues, but the fate of porcine parvovirus will be tested in future field-scale trials of bioreduction.

Campylobacter spp. was predicted to be absent from all samples due to its penchant for microaerophilic conditions. Indeed, temporary technical issues resulted in a brief reduction in aeration rate on day 23 which corresponds with an observed increase in *Campylobacter* spp. numbers; thereafter numbers reduced considerably and none were recovered at the latter stages of the trial (Fig. 3B). TVC values were initially lower in

inoculated MBVs than the sum of both *Salmonella* and *E. faecalis*; though this is likely to reflect the differences in the nature of the agars and plating methods used.

Williams et al. (2009) hypothesised that microbial competition and predation reduced the population of pathogens within bioreduction vessels. Numerous other studies have showed the reduction of pathogens (including *S.* Senftenberg) in a range of wastes is promoted by competition from naturally present antagonistic microbes (Ceustermans et al., 2007; Hussong et al., 1985; Pietronave et al., 2004; Sidhu et al., 2001). However, some species of *Enterococcus* are able to produce bacteriocins that give them a competitive edge over other organisms (Fisher and Philips, 2009). Together with its ability to survive a wide range of environmental conditions (Fisher and Philips, 2009), this may explain why *E. faecalis* could still be recovered at the end of the trial; although it too may have decreased further had the trial period been extended.

Many pathogens can enter a viable but non-culturable state (VBNC) when under environmental stress and this may lead to underestimation of numbers when using culturing methods. However, bacteria containing the *lux* gene that have entered a VBNC state can still be detected in real-time by measuring bioluminescence (Duncan et al., 1994; Ritchie et al., 2003). Luminescence directly reports on bacterial metabolic activity which represents a prerequisite for host infection (Unge et al., 1999). Bioluminescence measurements showed that there was a concomitant decrease in both numbers and metabolic activity of *E. coli* O157; hence conditions within the vessels were not conducive to the organism's proliferation. Although currently not widely available, further trials with additional constructed *lux*-marked pathogens would be worthwhile to determine the effects of bioreduction on a greater range of target organisms.

Only low levels of bioaerosols were detected from the simulated bioreduction systems. Andersen samplers recover only the culturable fraction of micro-organisms and various molecular techniques have occasionally been shown to be useful in analysing bioaerosols in different environments (Fallschissel et al., 2009; Junhui et al., 1997; Maron et al., 2005). However, Andersen samplers are the preferred industry choice for sampling bioaerosols from other types of organic wastes (Stagg et al., 2010), and the large volume of air processed at each sampling point was deemed more than sufficient to detect the presence of bioaerosols. Our findings support others studies (Adkin et al., 2010) that propose bioreduction provides negligible risk of hazardous bioaerosol generation.

Where possible, more than one strain or serotype of each pathogen was added to the simulated bioreduction systems so as to negate potential inter-strain variation in survival. Whilst natural strains of pathogens may show greater resistance to environmental stresses than experimental cultures, it is unlikely that natural populations of the microaerophilic organisms or facultative anaerobes such as those used in this study would be any better adapted to cope with the process of forced aeration and other stresses encountered within a bioreduction system (Williams et al., 2009). This was also evident in our results as survival patterns of introduced strains were similar to natural strains within controls for all micro-organisms. However, molecular methods (e.g. automated ribosomal intergenic spacer analysis fingerprinting) may differentiate between strains (Cardinale et al., 2004) and therefore could be used to elucidate such points in future trials.

The rate of carcass degradation is likely to be largely governed by biological activity and the composition of the microbial community. Utilising molecular techniques may elucidate the changes in microbial population and whether such changes affect the

rate of degradation. Such information may be valuable in developing novel biological catalyst products to further improve the process. Further work at field-scale is also necessary to validate the energy demand of bioreduction and to identify where gains in efficiencies could be made.

5. Conclusions

This work indicates that bioreduction is efficient at containing pathogens from carcass material and hence that the system could potentially be suitably secure to store fallen stock prior to ultimate disposal. Further investigation at field-scale level that also includes other relevant organisms (e.g. indicator viruses) is required so that the system can be soundly considered for industry use and incorporation into the revised EU Animal By-Products Regulations (1069/2009).

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Table 1: Detection of bioaerosols from the inoculated mini bioreduction vessels (CFU m^{-3}). Values represent means ± standard error of the mean. ND denotes not detected.

Day	*Salmonella* spp.	*E. faecalis*	*E. coli* O157	*Campylobacter* spp.	Total Viable Counts
0	31.9 ± 21.2	15.6 ± 10.9	ND	ND	15.7 ± 6.3
24	ND	4.4 ± 4.4	ND	ND	147.8 ± 126.7
57	ND	1.1 ± 1.1	ND	ND	2925.6 ± 2917.2
85	ND	ND	ND	ND	41.3 ± 31.3

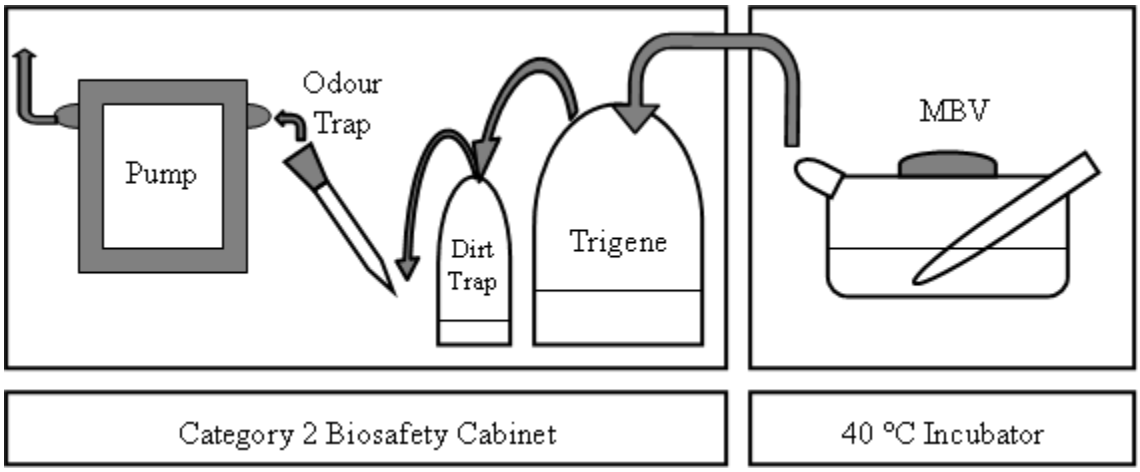

Figure 1: Experimental layout of the mini bioreduction vessels (MBV). Each MBV was attached to a large disinfection Trigene bottle via silicone tubing with the exception of the second control MBV which was attached to its own Trigene bottle and vacuum pump.

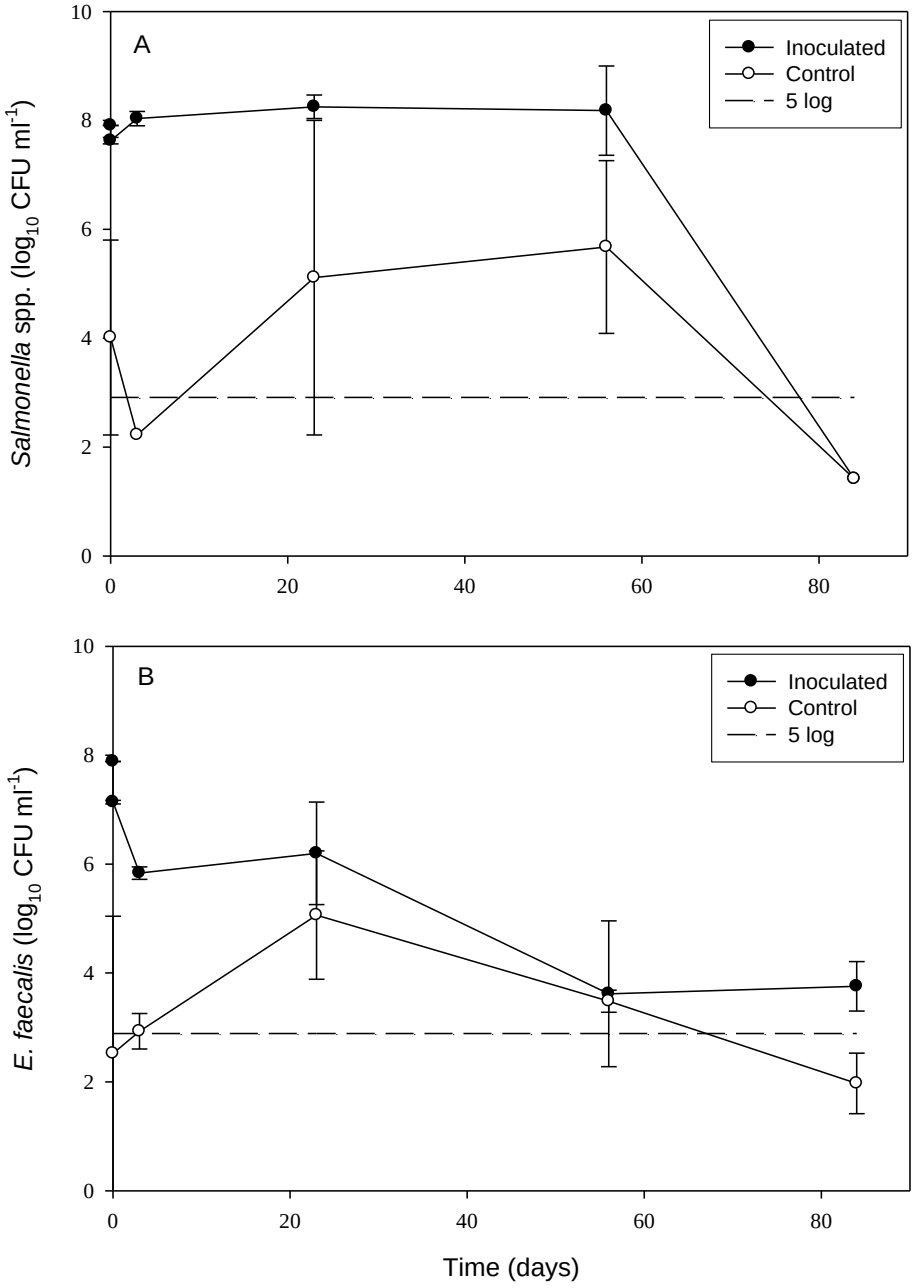

Figure 2: Changes in numbers of *Salmonella* spp. (A) and *Enterococcus faecalis* (B) over time in the inoculated and control mini bioreduction vessels. The dashed line represents a 5-log reduction in numbers from the original starting concentration. Values represent means ± standard error of the mean.

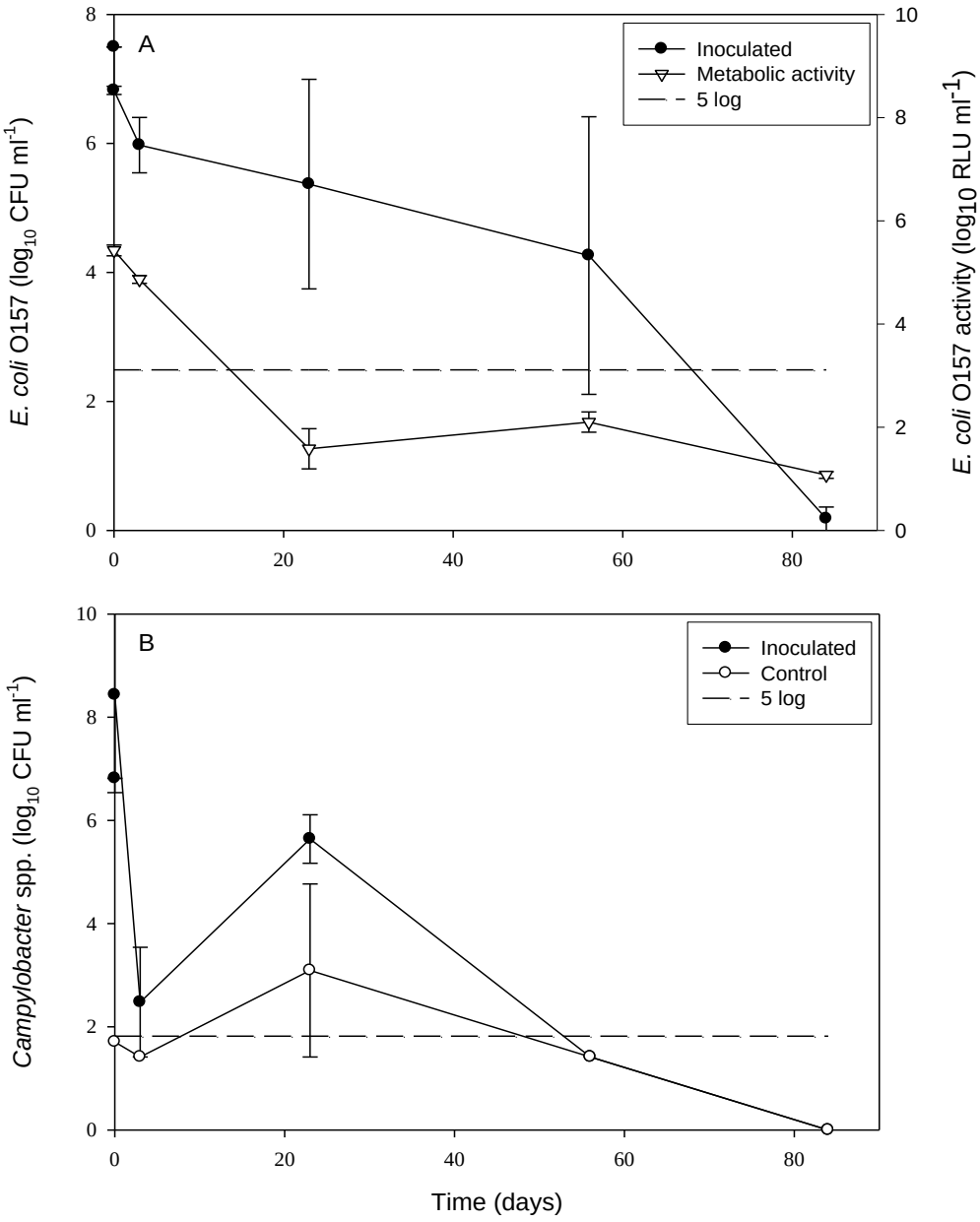

Figure 3: Changes in numbers and metabolic activity (as measured by relative light units of luminescence) of *Escherichia coli* O157 in the inoculated mini bioreduction vessels (A) and numbers of *Campylobacter* spp. (B) over time in the inoculated and control mini bioreduction vessels. The dashed line represents a 5-log reduction in numbers from the original starting concentration. No *E. coli* O157 was present in the controls. Values represent means ± standard error of the mean.

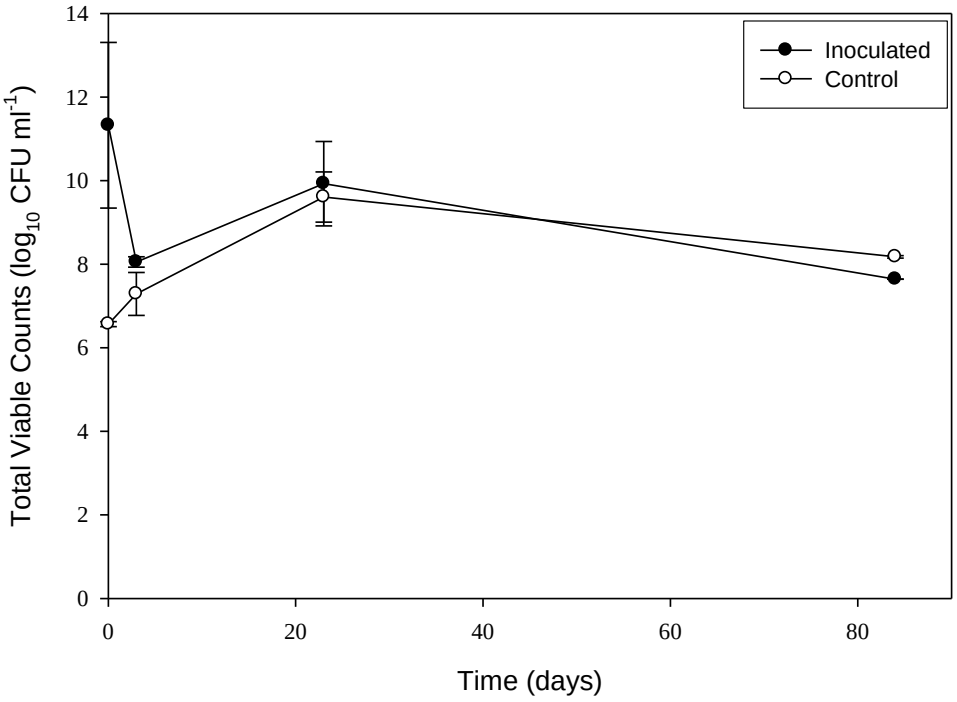

Figure 4: Changes in numbers of Total Viable Counts on standard plate count agar over time in the inoculated and control mini bioreduction vessels. Values represent means ± standard error of the mean.