

**Bangor University**

## **DOCTOR OF PHILOSOPHY**

### **Bioreduction : an alternative strategy for storing fallen stock prior to disposal**

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Bioreduction: An alternative strategy for storing fallen  
stock prior to disposal

**Ceri Gwyther**

October 2012



PRIFYSGOL  
**BANGOR**  
UNIVERSITY

A thesis submitted in fulfilment of the requirements of a  
Doctor of Philosophy

School of Environment, Natural Resources and Geography

## SUMMARY

Bioreduction has been proposed as an on-farm storage facility for fallen stock prior to final disposal in accordance with the Animal By-Products Regulations, ABPR (EC 1069/2009). In order for bioreduction to be approved under the legislation it must be shown to be biosecure. Therefore, the main aim of this thesis was to assess the risk of pathogen proliferation in the liquor and bioaerosols generated under operational and simulated breakdown scenarios. Secondary objectives consisted of improving the technology and included: determining the efficacy of commercial accelerants in catalysing the bioreduction process; the use of the carbon footprint to identify potential environmental improvements in future vessel designs; and identifying the main physicochemical parameters and enzyme activity associated with bioreduction, in order to more fully understand the underlying biodegradation processes occurring within the vessels.

A range of microbiological and molecular techniques were employed to analyse pathogen survival and assess microbial communities and included; traditional culturing, bioaerosol analysis, 16S rRNA sequencing and automated ribosomal intergenic spacer analysis (ARISA). Carbon footprints were analysed using ISO14040 Life Cycle Assessment guidelines, greenhouse gas emissions using a portable gas meter and physicochemical and enzyme assays using standard techniques, often based on soil or compost protocols in the absence of specific bioreduction methods.

Whilst there is always room for vessel design improvement such as using solar energy and determining loading capacity to reduce foaming, the technology has repeatedly shown to reduce the volume of waste to be ultimately disposed and has gained favour within the livestock industries. Bioreduction has also shown to be biosecure in both laboratory and field settings and under both optimal and sub-optimal conditions. The lack of pathogen proliferation and dispersal meets the requirements of the ABPR for the storage of fallen stock. Therefore, it is recommended that the regulations are updated to include bioreduction for both pig and sheep carcasses.

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## LIST OF PUBLICATIONS

This thesis is based on the following articles and manuscripts. Each is referred to in the following text by the corresponding article number.

**Article 1: Gwyther, C.L., Williams, A.P., Golyshin, P.N., Edwards-Jones, G., Jones, D.L.** 2011. The environmental and biosecurity characteristics of livestock carcass disposal methods: A review. *Waste Management*, 31, 767-778.

**Article 2: Gwyther, C.L., Jones, D.L., Golyshin, P.N., Edwards-Jones, G., Williams, A.P.** 2012. Fate of pathogens in a simulated bioreduction system for livestock carcasses. *Waste Management*, 32, 933-938.

**Article 3: Gwyther, C.L., Jones, D.L., Golyshin, P.N., Edwards-Jones, G., McKillen, J., McNair, I., McDonald, J.E., Williams, A.P.** Bioreduction of sheep carcasses effectively contains and reduces pathogen levels under operational and simulated breakdown conditions. *Submitted for peer review*

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Finally, my thanks go out to those who gave their lives for this thesis – sheep and pig alike, you made the ultimate sacrifice for the greater good. Can there be any better cause?



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## **ABBREVIATIONS**

<b>ABPR</b>	Animal By-Products Regulations
<b>ARISA</b>	Automated ribosomal intergenic spacer analysis
<b>BPEX</b>	British Pig Executive
<b>BV</b>	Bioreduction vessel
<b>CF</b>	Carbon footprint
<b>CFU</b>	Colony forming units
<b>CO<sub>2</sub>e</b>	Carbon dioxide equivalents
<b>CONBV</b>	Control bioreduction vessel
<b>CPE</b>	Cytopathic effect
<b>EFSA</b>	European Food Safety Authority
<b>EC</b>	European Commission
<b>EU</b>	European Union
<b>FMD</b>	Foot and mouth disease
<b>GHG</b>	Greenhouse gas
<b>HCC</b>	Hybu Cig Cymru, the meat promotion service in Wales
<b>LCA</b>	Life cycle assessment
<b>MBV</b>	Mini bioreduction vessel (lab-scale)
<b>MUF</b>	Methylumbelliferyl
<b>NTU</b>	Nephelometric turbidity units
<b>PCR</b>	Polymerase chain reaction
<b>PPV</b>	Porcine parvovirus
<b>qPCR</b>	Quantitative polymerase chain reaction
<b>RLU</b>	Relative light units
<b>SSC</b>	Scientific Steering Committee
<b>SRM</b>	Specified risk material
<b>TCID<sub>50</sub></b>	The amount of a pathogenic agent that will produce a pathological change in 50% of the cell cultures inoculated
<b>TSE</b>	Transmissible spongiform encephalopathy
<b>TVC</b>	Total viable counts
<b>VBNC</b>	Viable but non-culturable
<b>WAG</b>	Welsh Assembly Government

## 1. Introduction

Agriculture is one of the UK's most important industries. In 2010, it covered 18.3 million hectares, approximately 76% of the total UK land area, and contributed £6.8bn to the economy (DEFRA, 2010). With close to 210 million animals in the UK, including cattle, sheep, pigs and poultry (DEFRA, 2010), routine mortality of a proportion of these animals is inevitable. Yet the biosecurity, environmental, economical, and social implications of this considerable waste stream has been understudied (Article 1).

The EU Animal By-Products Regulations (ABPR) determine the fate of routine mortalities and has restricted disposal options to incineration, rendering, alkaline hydrolysis, hunt packs and maggot farms (Anon, 2009). The regulations reduced the number of available on-farm disposal options available by removing the traditional methods of livestock disposal; namely burial and burning. It was feared that burial and burning were not sufficient treatment to remove prions, the agents responsible for transmissible spongiform encephalopathies (TSEs) (Anon, 2009). However, biosecurity concerns have been raised in the livestock industry over the removal of on-farm disposal options (Bansback, 2006) and there is evidence to suggest that not all farmers are complying with the regulations (Kirby et al., 2010). Nonetheless, the ABPR does allow for the development of novel disposal and storage options providing that scientific evidence can show the biosecurity benefits of the system (Anon, 2009). For *disposal* methods, this means that by the end of the process, indicator organisms such as thermotolerant bacteria must show a 5-log reduction in numbers, and thermotolerant viruses, a 3-log reduction (Bohm, 2008). Less stringent requirements are needed for the approval of novel *storage* systems (to be used in conjunction with ultimate disposal via an approved process); indicator organisms must not be able to proliferate as a result of the storage process prior to ultimate disposal (Anon, 2009).

Bioreduction is one such novel on-farm storage system. Williams et al. (2009) completed a preliminary study on the bioreduction of sheep carcasses. They defined bioreduction as being the “aerobic biodegradation of animal by-products in a partially sealed vessel [containing water], where the contents are mildly heated and aerated” (Williams et al., 2009). In all bioreduction experiments to date, a mesophilic temperature of 40 °C has been used. Carcasses are inputted into the vessel as and when they die on-farm. An incision is made into the abdomen to release the internal microorganisms and enzymes which will ultimately bioreduce the carcasses (Williams et al., 2009). The water in the vessels must cover two thirds of the carcass at all times to prevent desiccation, and a commercial

accelerant is used to catalyse the initial stages of decomposition (Gutiérrez et al., 2003). The liquor produced is moderately basic and nutrient-rich and can be removed from the vessel for disposal using standard waste suction pumps. Liquor must be disposed of as Category 1 waste under the ABPR (Williams et al., 2008, 2009); i.e. either by incineration or rendering (Anon, 2009). A proportion of the liquor is kept in the vessels as a starter inoculum for future bioreductions (Gutiérrez et al., 2003). In a twelve month trial involving sheep carcasses, Williams et al. (2009) found that the volume of carcasses that ultimately needed to be disposed was considerably reduced.

Calls have been made from the livestock industry in the UK, particularly from the sheep and pig sectors, for novel storage technologies that will be situated on-farm (Bansback, 2009). Early work into bioreduction has shown that it is efficient at storing and reducing the volume of carcasses to be ultimately disposed (Williams et al., 2009). It is also a straightforward technology that requires little maintenance and therefore, from a practical standpoint, bioreduction is an ideal alternative to existing disposal options such as the central collection service or on-farm incineration.

## **2. Aims**

The following thesis seeks to address some of the knowledge gaps around bioreduction and looks at the biosecurity impacts, environmental and practical issues; and tries to define the biochemical and microbial processes involved in the bioreduction process. The preliminary aim of this thesis is to provide a more comprehensive analysis of the fate of microbial indicator organisms in both the liquor and bioaerosols produced during the bioreduction of carcasses. If applicable, this information will be used as the basis of an application to the EU for the legalisation of bioreduction as a novel *storage* option. Secondary aims include:

- determination of the efficacy of the commercial accelerant;
- to use the carbon footprint of current fallen stock disposal options, including bioreduction, to highlight areas for future environmental improvements, in line with Welsh Government objectives (WAG, 2009); and,
- to determine important processes in the biochemical cycles during decomposition in order to better understand how bioreduction works.

### **3. Outline of the thesis**

The majority of work on bioreduction so far has been on sheep carcasses (Williams et al., 2009). Therefore, some of the work in this thesis has continued to focus on this species in order to collate a substantial information base. However, work on other notable species is also recommended if bioreduction is to be taken up by the wider farming community. Therefore, two articles have been based on the bioreduction of pig carcasses.

To address the aims outlined in Section 2, this thesis has been divided into six articles. The first article is a review of current literature on existing methods of livestock disposal and outlines novel storage options such as bioreduction (Gwyther et al., 2011). The following two articles aim to address the fate of pathogens during bioreduction. A preliminary laboratory study is outlined in Article 2 (Gwyther et al., 2012) and a field-scale ratification of these results is shown in Article 3, which involved two field trials. The first was run under standard operating conditions and the second trial was run under simulated breakdown conditions (Article 3) as this was necessary according to the European Food Safety Authority (EFSA), the committee responsible for assessing the legality of new disposal and storage options (EFSA, 2008). The efficacy of the commercial accelerant and others available on the market was examined in Article 4. The carbon footprint of bioreduction was compared against a number of other disposal and storage options in Article 5, along with the environmental implications of final disposal, i.e. incineration, rendering or the use of bioreduction liquor as a fertiliser. Finally, Article 6 investigates the biochemical cycles within the bioreduction vessels by assessing enzyme activity and microbial communities and is an extension of the work undertaken by Williams et al. (2009). The results and conclusions of these articles are discussed in detail in each article and summarised in section 5.

## 4. Methods

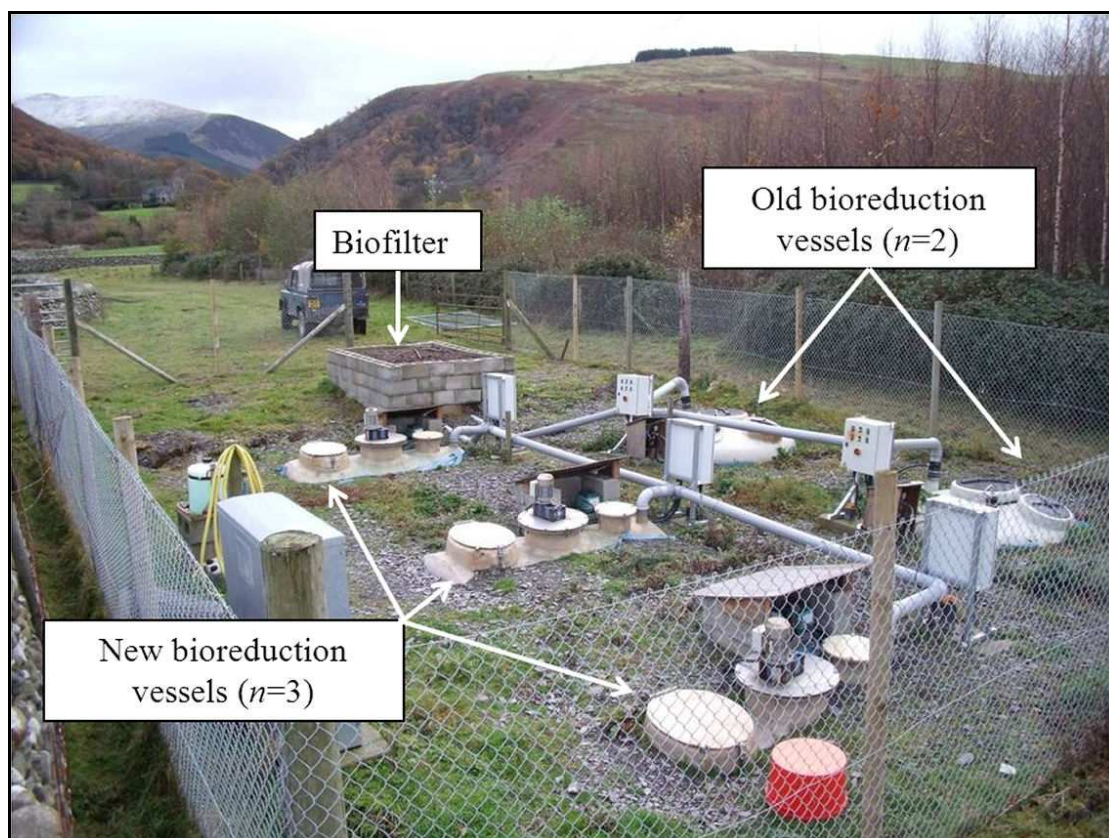
All experimental analyses were carried out at Bangor University's Environment Centre Wales (ECW) and Henfaes Research Station. A detailed explanation of the experimental design, sample collection and analysis are found within each article. However, a brief summary of the field site, bioreduction vessels and laboratory-scale vessels is outlined below.

### 4.1. Field studies

#### 4.1.1. Study site and bioreduction vessel description

Field trials were undertaken at Bangor University's Henfaes Research Station (53°14'05"N, 4°00'50"W). The field study site consisted of 5 bioreduction vessels in total (Fig. 1). Two of these were the vessels used in the original trial by Williams et al. (2009) (Fig. 2A). The remaining three were of a slightly different design but with the same volumetric capacity, being horizontally rather than vertically orientated (Fig. 2B). In addition, the new vessels had a built-in agitator to stir the carcasses (Fig 2C); although these remained switched off during all trials to keep consistency with previous work. Each bioreduction vessel had a 6,500 l capacity and was made from a combination of a high density polymer and thermostable glass fibre, and coated inside with a biphenolic-type resin (Gutiérrez et al., 2003). An oil-filled element was heated using electricity to regulate the water temperature, whilst air was pumped into the vessel at a pressure of 50 kPa for 45 min h<sup>-1</sup> to provide an aerobic environment (Williams et al., 2009). Vessels were filled with an appropriate amount of water (approximately 3,000 l) and brought to temperature (40 °C) before the addition of carcasses. All bioreduction vessels were linked to a compost and woodchip biofilter through silicon tubing (Fig. 1). The vessels had previously relied on activated charcoal filters in the chimneys to reduce odours and emissions (Williams et al., 2008), but due to clogging problems, the new biofilter was constructed.





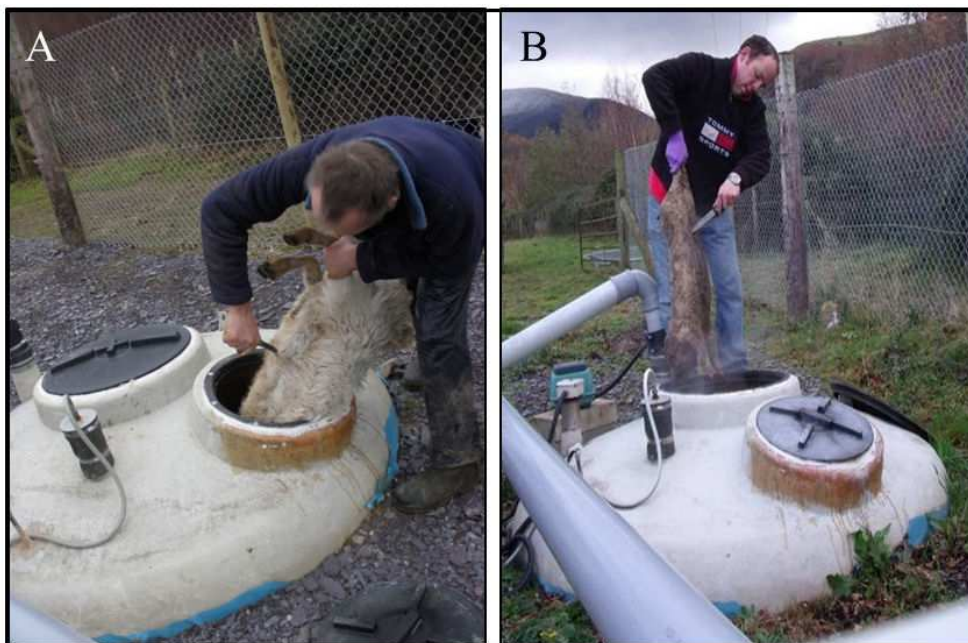
**Figure 1:** The layout of bioreduction vessels at the study site

#### 4.1.2. *The addition of carcasses*

Both sheep and pig carcasses were used in the work that led to this thesis. Permission was received from the Bangor University's Ethics Committee before experiments took place. Whenever possible, fallen stock were used; either from Henfaes Research Centre's 1,600 breeding ewe flock or the neighbouring pig farm. Where necessary, animals were euthanised using approved practices. As described in the Introduction (Section 1), carcasses received an incision into the abdomen to release the internal microorganisms and enzymes which bioreduced the carcasses (Fig. 3). Sheep carcasses, or parts thereof, were used in Articles 2 and 3 whilst pig carcasses, or parts thereof, were used in Articles 4 and 6.



**Figure 2:** Photographs of the old, or vertical, (A) and new, or horizontal, (B) bio-reduction vessels and the inside of one of the horizontal bio-reduction vessels showing the agitator, or mixing paddle (C).



**Figure 3:** An incision is made into the abdomen of both sheep (A) and pig (B) carcasses before inputting into the vessels.

## *4.2. Laboratory studies*

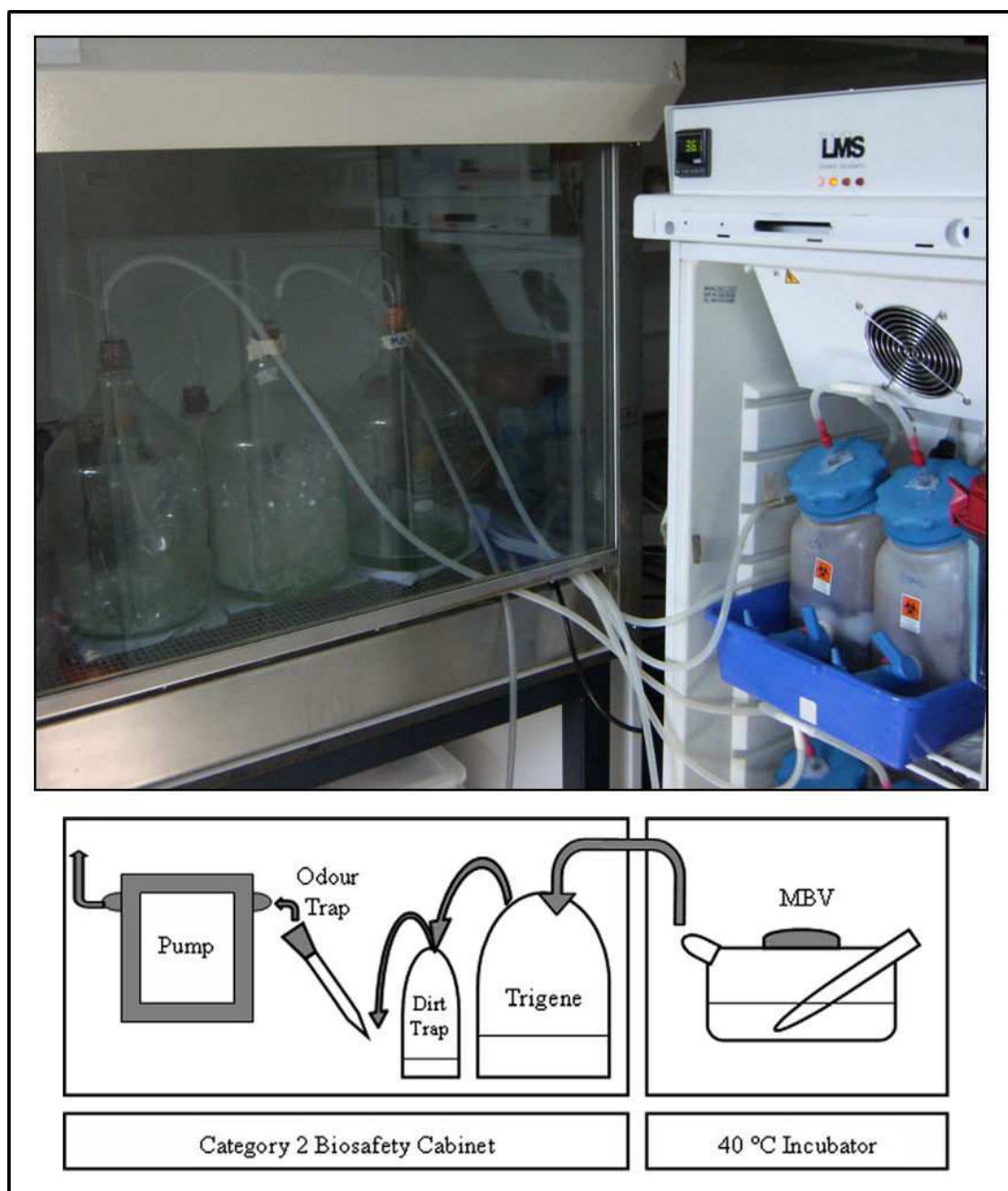
### *4.2.1. Laboratory-scale bioreduction vessels*

A laboratory-scale bioreduction vessel was used in Article 2. This consisted of a series of 5 L polypropylene containers (19 cm high × 13 cm wide × 26 cm long) aerated by vacuum pumps to recreate the level of aeration observed in the bioreduction vessels (Fig. 4). A total of five mini bioreduction vessels (MBVs) were used in total; two as controls and three inoculated with pathogens. Each pair of MBVs was attached by silicone tubing to a bottle containing commercial disinfectant (Trigene; Medichem, UK), a dirt trap and an activated charcoal odour filter. To prevent contamination of the laboratory by potentially dangerous bioaerosols, all vessels vented to a biosafety cabinet. The MBVs were placed in a darkened incubator set to 40 °C (Fig. 4). Due to the complications associated with the vacuum pump system, and the space requirements, a much simpler laboratory-scale vessel was used in the accelerants trial in Article 4. In this instance, 50 ml tubes were shaken in an orbital shaker at 150 rev min<sup>-1</sup> to keep the liquor aerated.

### *4.2.2. The addition of carcass components*

As it was not possible to use whole carcasses in the laboratory-scale experiments, carcass components were used to replicate as much of the whole carcass as possible. Annex 1 outlines the proportions and quantity of materials used in Article 2. Again, methods used in Article 4 were simplified. The carcass material in this case was from pork chops and consisted of only lean meat.





**Figure 4:** Experimental layout of the laboratory-scale bioreduction vessels used in Article 2.

# Article 1

## The environmental and biosecurity characteristics of livestock carcass disposal methods: A review

**Published:** Gwyther, C.L., Williams, A.P., Golyshin, P.N., Edwards-Jones, G., Jones, D.L. 2011. The environmental and biosecurity characteristics of livestock carcass disposal methods: A review. *Waste Management*, 31, 767-778. DOI: 10.1016/j.wasman.2010.12.005

**Notes on the authorship:** C.L Gwyther did the research and C.L. Gwyther and A.P. Williams wrote the manuscript with critical discussion and revision by co-authors.

# Article 2

## Fate of pathogens in a simulated bioreduction system for livestock carcasses

**Published:** Gwyther, C.L., Jones, D.L., Golyshin, P.N., Edwards-Jones, G., Williams, A.P. 2012. Fate of pathogens in a simulated bioreduction system for livestock carcasses. Waste Management, 32, 933-938. DOI: 10.1016/j.wasman.2011.10.031

**Notes on the authorship:** C.L. Gwyther, D.L. Jones, A.P. Williams and G. Edwards-Jones planned the research. C.L. Gwyther carried out the experimental work. C.L. Gwyther and A.P. Williams wrote the manuscript with revision by co-authors.

# Article 3

## Bioreduction of sheep carcasses effectively contains and reduces pathogen levels under operational and simulated breakdown conditions

**Submitted for peer review:** Gwyther, C.L., Jones, D.L., Golyshin, P.N., Edwards-Jones, G., McKillen, J., McNair, I., McDonald, J.E., Williams, A.P. Bioreduction of sheep carcasses effectively contains and reduces pathogen levels under operational and simulated breakdown conditions.

**Notes on the authorship:** C.L. Gwyther, A.P. Williams, D.L Jones and G. Edwards-Jones planned the research. C.L Gwyther carried out the experimental work. J. McKillen and I. McNair quantified the virus and C.L. Gwyther and J.E. McDonald analysed the 16S rRNA sequences. C.L Gwyther and A.P. Williams wrote the manuscript with revision by co-authors.

# Article 4

## The efficacy of various commercial accelerants at enhancing the bioreduction of carcass constituents

**Unpublished:** Gwyther, C.L., Adams-Huset, A., Jones, D.L., <sup>†</sup>Edwards-Jones, G., Williams, A.P. The efficacy of various commercial accelerants at enhancing the bioreduction of carcass constituents.

**Notes on the authorship:** C.L. Gwyther, Adams-Huset, and A.P. Williams planned the research. C.L. Gwyther carried out the experimental work. C.L. Gwyther wrote the manuscript with revision by co-authors.

<sup>†</sup>G. Edwards-Jones was involved in the original grant proposal and conceptual design of this PhD and therefore is credited posthumously.



# Article 5

## A comparison of the carbon footprints of conventional and novel systems for the storage and disposal of animal carcasses

**To be submitted for peer review:** Gwyther, C.L., Jones, D.L., Norton, A. Edwards-Jones, G., Williams, A.P. A comparison of the carbon footprints of conventional and novel systems for the storage and disposal of animal carcasses.

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# Article 6

## Characterisation of physicochemical cycling and enzyme activity during the bioreduction of pig carcasses

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# Appendices

- Appendix I:** The quantity of carcass components used in Article 2
- Appendix II:** The physicochemical parameters analysed during the pathogen inoculation trials (Articles 2 and 3)
- Appendix III:** Optimising the enzyme assay

**The environmental and biosecurity characteristics of livestock carcass disposal methods: A review**

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## **ABSTRACT**

Livestock mortalities represent a major waste stream within agriculture. Many different methods are used throughout the world to dispose of these mortalities; however within the European Union (EU) disposal options are limited by stringent legislation. The legal disposal options currently available to EU farmers (primarily rendering and incineration) are frequently negatively perceived on both practical and economic grounds. In this review, we assess the potential environment impacts and biosecurity risks associated with each of the main options used for disposal of livestock mortalities in the world and critically evaluate the justification for current EU regulations. Overall, we conclude that while current legislation intends to minimise the potential for on-farm pollution and the spread of infectious diseases (e.g. Transmissible Spongiform Encephalopathies, bacterial pathogens), alternative technologies (e.g. bioreduction, anaerobic digestion) may provide a more cost-effective, practical and biosecure mechanism for carcass disposal as well as having a lower environmental footprint. Further social, environmental and economic research is therefore warranted to assess the holistic benefits of alternative approaches for carcass disposal in Europe, with an aim to provide policy-makers with robust knowledge to make informed decisions on future legislation.

*Keywords:* Animal disease; composting; greenhouse gases; prions; viruses; zoonoses.

## 1. Introduction

Routine mortality of animals is an inevitable consequence of livestock farming systems. With a global livestock population of approximately  $1.9 \times 10^{10}$  birds and  $2.31 \times 10^8$  mammals (FAO, 2007), farming systems generate a significant volume of mortalities that need to be disposed of safely, practically and economically. Throughout history, the most widely utilised methods for disposal of on-farm mortalities has probably been burial and to a lesser extent, burning. However, implementation of the European Union (EU) Animal By-Product Regulations (1774/2002) (Anon, 2002) forbids these practices within the EU and limits the disposal routes to incineration (either on or off-farm), rendering, high temperature / pressure alkaline hydrolysis, disposal at maggot farms or through licensed waste collectors (Anon, 2002). The prohibition within the regulations was founded on the perceived risk of pathogens and infective agents entering the animal feed chain due to their incomplete destruction during burial and burning of mortalities (Anon, 2002). Particular concern relates to the safe management of prions responsible for Transmissible Spongiform Encephalopathy (TSE) (Anon, 2002). However, carcass disposal is also perceived to be synonymous with pollution, such as the increased concentrations of soluble nitrogen in soil and groundwater due to burial (Ritter & Chirnside, 1995), odour issues or the fear of dioxins and furans being released into the air as a result of incomplete or uncontrolled combustion (Scudamore et al., 2002). It is therefore essential that disposal methods can eliminate or contain these risks. However, practices such as burial are still widely utilised outside of the EU (Anon, 2007). The different interpretation of the threats and/or risks posed by each disposal option raises questions about the quality of the evidence-base upon which legal decisions have been made. There is therefore a need to critically assess the biosecurity and pollution merits and drawbacks of the different disposal options currently available to farmers.

The following review outlines the major routine disposal routes used throughout the world and the biosecurity and environmental credentials of each. It also highlights areas where, due to a lack of peer-reviewed science, regulations have been obliged to make assumptions about the risks associated with particular disposal methods; particularly in the context of EU regulations. An analysis of the economic viability of each option is discussed briefly but a full economic analysis is beyond the scope of this review due to the lack of sufficient data and a fundamental difference in the respective cost of each method in different countries.

## **2. Current methods for disposal of livestock mortalities**

### *2.1. Burial*

The traditional methods of on-farm burial of livestock mortalities include burial in graves, trenches, or in open-bottomed containers referred to as mortality or disposal pits (CAST, 2008a; Freedman & Fleming, 2003). Livestock burial has been banned in the EU due to fears that infectious agents may inadvertently enter both the human food and animal feed chains or lead to environmental pollution (Anon, 2002). Outside of the EU, some concern has been raised that improper burial may lead to contamination of ground and surface water with pathogens and the chemical products of decomposition (NABC, 2004). However, no studies could be found that reported any serious environmental impact from routine disposal via burial. Indeed, Ritter and Chirnside (1995) concluded that the pollution from burial pits was similar to that of domestic septic tanks and could be controlled with legislation synonymous with on-site wastewater treatment regulation.

Many of the assumptions about the environmental impact of the burial of fallen (dead) stock have been made following mass-burial at incidences of high mortality. However, it is unlikely that the findings of such studies provide an accurate representation of the typical risks posed by routine burial of on-farm mortalities. For instance, weekly disposal of dead animals from an American turkey farm typically equates to approximately 2,000 kg (CAST, 2008a), whereas Glanville (2000) evaluated the environmental impact of burying 28,000 kg of turkeys in two pits following a barn ventilation failure. Similarly, numbers of dead sheep from a typical European farm will be significantly less than those generated following mass-disease outbreaks. During the UK Foot and Mouth Disease (FMD) outbreak in 2001, approximately 61,000 tonnes of carcasses were disposed of at four mass burial sites (Anderson, 2002). It is inevitable that such mass burial would pose considerably greater environmental and biosecurity risk than burial of routine mortalities and hence extrapolation of the results from studying such extreme events may be erroneous. Indeed, Vinten et al. (2008) concluded that the concentrations of *E. coli* and *Cryptosporidium* in ground and surface water were affected to a greater extent by excretion from live animals than they were from the burial of a small number of carcasses. The risk posed by routine burial should therefore be balanced against other widespread agricultural practices (e.g. farm waste land-spreading) so that the threat is realistically evaluated in relative terms.

In addition to the potential introduction and subsequent survival of pathogenic bacteria in soil and water arising from carcass burial, concern has also arisen that burial may lead to propagation of pathogens and subsequent pollution of groundwater and drinking water. Many factors affect the movement of pathogens through soil to groundwater, including soil type, permeability, water table depth and rainfall (Beal et al., 2005). However, adsorption, filtration and predation by natural microbial populations significantly reduce the amount of pathogens that eventually reach underlying groundwaters (Beal et al., 2005). Within an aquifer, there are also many factors that govern the inactivation of the pathogens, e.g. pH, water flow rate and substrate grain size (John & Rose, 2005). Taking all these factors into account, it is plausible that the numbers of pathogens reaching any drinking water source due to routine burial are likely to be low; particularly if boreholes and wells are deep, thereby increasing the time taken by pathogens to reach the underlying aquifer and thus the likelihood of their demise before reaching the water. In support of this, Myers et al. (1999) reported low concentrations of coliforms and *Salmonella* in observation wells surrounding disposal pits, concluding that bacteria did not move more than 30 m laterally in groundwater. Similarly, in a survey of poultry disposal pits, Ritter and Chirnside (1995) found the average concentrations of faecal coliforms and faecal streptococci in water samples to be relatively low (24 CFU 100 ml<sup>-1</sup> and 3 CFU 100 ml<sup>-1</sup>, respectively); with many samples testing negative. Indeed, no studies have been reported in the literature linking the burial of animal carcasses to detrimental effects on either human or animal health, although burial of humans within a water table has led to incidences of contaminated groundwater (Bastianon et al., 2000). Furthermore, the addition of hydrated lime (Ca(OH)<sub>2</sub>) to the base of burial pits has been shown to effectively reduce the survival of pathogens and the possibility for off-site pathogen transfer (Sanchez et al., 2008). The use of a chemical barrier to minimise risk is supported by Avery et al. (2009) who found no viable *E. coli* O157 cells in contaminated abattoir waste treated with lime applied at a rate of 10 g of CaO lime l<sup>-1</sup> waste. Applying lime both during the construction and subsequent operation of burial sites may impede the growth of all micro-organisms and hence slow the process of decomposition. However, in the context of improving biosecurity, it is a simple and cost-effective procedure that would be accessible to many farmers; justifying the case for further research to enable the scientific basis of current legislation to be critically evaluated.

Despite the seemingly low incidence of drinking water contamination with enteric pathogens arising due to burial of carcasses, some infectious material such as anthrax spores or prions can reside within the soil after carcass decomposition (Brown, 1998; Johnson et al.,



2006, 2007; Nechitaylo et al., 2010). This may lead to animals inadvertently ingesting contaminated soil and the infectious agents and hence may lead to development of neurodegenerative disease (e.g. BSE or scrapie) in the case of prions (Johnson et al., 2007), or the reintroduction of anthrax (Sharp & Roberts, 2006). While such events pose real risks, measures can be implemented to reduce the risk of prion transmission and propagation arising through burial of carcasses. Primarily, animals suspected of dying from neurodegenerative disease or anthrax should be automatically sent for incineration or rendering following examination by a veterinary practitioner. Burial sites could also be located away from livestock fields and at sufficient depth so that the potential for transfer of infectious agents back to the surface (e.g. through earthworm activity (Nechitaylo et al., 2010; Williams et al., 2006)) is very low. Indeed, burial of carcasses at depth may stimulate prion-degrading enzyme production by indigenous microbial populations, thus further reducing any threat (Rapp et al., 2006). The use of soil additives incorporating prion degrading proteases or microbes known to degrade prions could also stimulate prion degradation and is a potential area for future research. Risk assessments undertaken in 1997 after the UK BSE crisis concluded that the leachate from the landfills used to dispose of BSE-infected cattle was not likely to cause a significant risk to local inhabitants (Spouge & Comer, 1997). However, burial at depth may induce hypoxic conditions, particularly in soils with very high moisture content (e.g. when waterlogged) (Killham, 1994; Pounder, 1995). This may impede microbial degradation and ultimately sustain infectivity and thus pose a biosecurity threat if pits are inadvertently exposed at a later date. Nevertheless, the associated probability of TSE transmission through burial of carcasses in Europe is clearly reduced given that the number of livestock infected with prions has decreased dramatically over the last decade (DEFRA, 2008a).

In the UK, groundwater vulnerability maps were used during the 2001 FMD outbreak to locate suitable mass-burial sites (Anderson, 2002) and are currently used to locate suitable human cemetery sites (EA, 2004). A similar risk assessment method could be employed to reduce the risk of contamination to groundwater from routine livestock burial using additional datasets, including locations of boreholes and wells, topography, and land-use. Such methods could identify potential on-farm burial sites that minimise the risk of environmental pollution whilst proving to offer a viable and practical option for farmers to dispose of on-farm mortalities. In summary, more evidence is needed to definitively test the environmental impact of burial of routine mortalities.

## *2.2. Burning*

On-farm burning of livestock mortalities on pyres is commonly used as a disposal method in many countries. Burning on pyres has also been used extensively in many disease outbreaks such as the 2001 FMD outbreak in the UK (Scudamore et al., 2002), and the 2004/2005 outbreak of anthrax in Uganda (Wafula et al., 2008). Despite the potential for pollution to occur from the mass-burning that occurred during the FMD outbreak, evidence of groundwater contamination from ash burial was minor, soil contamination from pyres was found to be negligible, and air emissions from pyres did not significantly affect air quality beyond the immediate vicinity (EA, 2001). Furthermore, studies indicated that the spread of FMD virus via smoke plumes was very unlikely (Champion et al., 2002). Biosecurity concerns therefore principally reside with the fate of TSEs, as open-air combustion is not likely to be as complete or reach as high a temperature as incineration, increasing the risk of TSEs remaining infectious (EC SSC, 2003a) (see Section 2.3). However, studies by Brown et al. (2004) suggest that the potential for the airborne or bottom ash transfer of TSEs from animal carcasses is highly unlikely. Further, complete combustion can be successfully achieved when sufficient labour, air and fuel is provided (Animal Health Australia, 2007).

Possible human health risks associated with on-farm burning (apart from physical burns and direct smoke inhalation) include the emission of dioxins from incomplete carcass combustion. Dioxins and furans are carcinogens and can negatively affect human reproduction, development and immune systems (Rier, 2008). Dioxins released from pyres during the 2001 FMD outbreak were estimated to be between 7 and 73% of total annual UK dioxin emissions (EA, 2001), yet there were no significant dioxin concentration increases in products destined for the food chain at that period (Rose et al., 2005). Although the environmental impact of burning was shown to be minimal, considerable social concerns were expressed regarding odour, unsightliness, etc. (Anderson, 2002; EA, 2001); so much so it resulted in the abolishment of pyre burning as a viable disposal option (Scudamore et al., 2002). Nevertheless, such conclusions were drawn following mass-burning at over 950 sites (EA, 2001) and it is unclear whether burning of routine on-farm mortalities would raise such concerns or pose any environmental risk if performed effectively. Indeed, there is little evidence to legitimately deny or endorse the use of on-farm burning for routine disposal and more scientific analyses of pyres should be instigated to test common conceptions (e.g. increased dioxin levels and groundwater contamination), especially as disposal on pyres could potentially be used again should another disease outbreak occur (Anon, 2002). Such

work should be supported by social studies to elucidate the fears and possible misconceptions associated with livestock burning so that effective communication of risk can occur.

### *2.3. Incineration*

Incineration is the process where animal carcasses or by-products are burnt at high temperatures ( $\geq 850$  °C) to produce an inorganic ash (Anon, 2002; NABC, 2004). The process is expected to destroy all infective agents (NABC, 2004). Ash typically represents 1 to 5% of initial carcass volume (Chen et al., 2003, 2004), though this will vary with the incinerator type, process, fuel and animal species. In EU countries, ash resulting from specified risk material (SRM) (e.g. the spinal cord and brain) is subsequently sent to designated landfill sites (in accordance to the ABPR), as is the recommendation in other countries such as the USA (NABC, 2004).

The principal concern with incineration of carcasses relates to gaseous emissions; however, small-capacity incinerators in some EU states have been deemed to be exempt from local air pollution controls as emissions of key pollutants represent  $\leq 0.2\%$  of the total air emissions (AEA Technology, 2002). Further reductions in harmful emissions may also occur after adoption of optimum techniques as introduced with the ABPR (e.g. use of afterburners).

Polycyclic Aromatic Hydrocarbon (PAH) emissions from two animal waste incinerators have been measured and directly compared with those from medical waste incinerators. Mean concentration of PAHs in the flue gas were greater in the animal incinerators than the medical waste incinerator, which was attributed to higher chamber retention times in the medical waste facility (Chen et al., 2003). In a further study, metal concentrations in the flue gas were found to be higher in the animal carcass incinerators than the medical incinerators (Chen et al., 2004). As neither of the two animal waste facilities met the ABPR (1774/2002) standards of heating to 850 °C for at least two seconds (Anon, 2002), yet the medical waste facility did, this suggests that current EU standards should reduce emissions from on-farm incinerators if operated correctly. However, more evidence is needed to elucidate the gaseous emissions arising from incineration of carcasses, especially under scenarios where the technology may not be working under optimal conditions.

Other health concerns arising from incineration include the release of dioxins and furans from flue gas and fly ash. There is a risk that dioxins and furans from incomplete combustion can settle in areas around carcass incinerators and could enter the food chain

through grazing animals or through human consumption of contaminated crops. However, afterburners fitted to incinerators can dramatically reduce the risk of noxious emissions release and numerous studies on different types of incinerators have found that dioxin and furan emission levels are rarely higher than ambient concentrations (Mari et al., 2008; Nadal et al., 2008; Yan et al., 2008). Furthermore, concentrations of dioxins and furans decrease significantly with increasing distance from incinerators (Yan et al., 2008) and the siting of on-farm incinerators is regulated within the EU (e.g. so as not to be within the immediate vicinity of livestock (Anon, 2002)). Indeed, it is thought that dioxins and furans from small animal incinerators account for only 0.07% of total UK dioxin emissions (AEA Technology, 2002). In theory, land-spreading of the generated ash as a soil improver may increase the risk of dioxins and furans entering the food chain via bioaccumulation. However, it is likely that this would pose an extremely low risk given the low concentrations released by small animal incinerators. With regards to human health, a study of large-scale municipal solid waste incinerators indicated elevated dioxin levels in operators who worked with bottom ash (Liu et al., 2008). However, further work is needed to elucidate if such effects occur from small-scale facilities.

There has been some debate previously about the effectiveness of incinerating TSE-infected carcasses and SRM (NABC, 2004). However, it is generally accepted that incineration destroys prion proteins more effectively than other methods of livestock disposal (with the possible exception of alkaline hydrolysis; (NABC, 2004)). Concerns have been raised about the levels of TSE remaining in the fly ash and slag generated, hence the requirement to land-fill all ash potentially infected with TSEs in the EU. Risk assessments have shown that there is less than a 1 in  $1 \times 10^9$  chance of the most exposed individual being infected with BSE via ingestion of ash following incineration and that the degree of infectivity of ash generated from incineration of BSE-infected meat and bone meal would be negligible (Spouge & Comer, 1997). The main risk to humans is attributed to the contamination of groundwater supplies from leaking sewerage pipes containing washwater from spillages of TSE-infected material at the incinerator (Spouge & Comer, 1997). In reality, the probability of this happening is extremely low, particularly if effluent is treated on-site. From a human and animal health perspective, the high temperature of incineration also completely destroys zoonotic and animal pathogens, including resilient spore-forming bacteria such as *Bacillus anthracis* (anthrax) (NABC, 2004). Land-spreading of ash from incineration of pigs and poultry is permitted in the UK, although under increasingly stringent

regulation (DEFRA, 2008b). Whilst land-spreading of ash derived from carcass incineration can potentially cause environmental damage (e.g. through heavy metal pollution (Chen et al., 2004)), a search of the literature failed to find any evidence which justifies the introduction of more stringent regulation. If such regulations become unworkable, it may result in the unnecessary land-filling of material that could be used in the fertiliser industry as a potential soil improver (Paisley & Hostrup-Pedersen, 2005).

One of the main perceived risks related to off-farm incineration is the transportation of dead livestock between farms. In Europe, centralised collection services exist for livestock mortalities where licensed operators collect carcasses and subsequently transfer the animals for incineration (or rendering) as necessary. It is inevitable that the vehicles may cover significant distances between farms whilst they are laden with carcasses from diseased animals and this has raised significant concerns within the livestock industry (Kirby et al., 2010). Such concerns appear to be justified as it was found that transporting animals between premises facilitated the spread of the FMD virus in the UK (Anderson, 2002; Scudamore et al., 2002); whilst transport of carcasses could propagate other serious animal diseases such as avian influenza (Pollard et al., 2008) and BSE (Spouge & Comer, 1997). The lag time between the death of a diseased animal and its collection may also pose a hazard if carcasses are not stored securely. It should be remembered that the risk of propagating disease via transporting carcasses between farms may be reduced given that some infective agents (e.g. viruses) survive only on live animals. Further, such risks may be reduced via employing good biosecurity practices such as disinfection of collection vehicles and protective clothing between sites; and by having sealed containers which livestock or vermin cannot access and which fluids cannot escape (Pollard et al., 2008). However, it is unlikely that such practices are always performed by all farmers and contracted operators, especially given the number of operators needed to run a national collection service. It is clear that further studies are needed to elucidate the risks of disease propagation through transport of carcasses both within and between farms.

Studies are required to directly compare the environmental footprint of incineration against other carcass disposal options via a life-cycle assessment (LCA) approach. Incineration of carcasses is likely to generate greenhouse gas (GHG) emissions due to the energy-intensive nature of the process and the relatively high water content of carcasses. The limited number of central incinerators also necessitates long-distance transportation of fallen stock, although this may be balanced against greater efficiency when larger waste volumes

are incinerated. There may therefore be an argument that due to biosecurity and environmental concerns, incineration should take place on-farm. Nevertheless, outside of the EU, on-farm incineration is not subject to the same monitoring regimes as commercial high-capacity sites and therefore may not be as stringently regulated as those in the EU.

#### *2.4. Rendering*

Rendering entails crushing carcasses and animal by-products into particles of a uniform size, heating the particles and then separating out the fat, proteinaceous material and water into, where possible, useful products including meat and bone meal and tallow (CAST, 2008a; Kalbasi-Ashtari et al., 2008; Woodgate & van der Veen, 2004). In the EU, mammalian meat and bone meal must now be land-filled, incinerated or used as a fuel source (Anon, 2002); although reductions in TSE levels may lead to it being reinstated as an additive for animal feed (Anon, 2010). Tallow from rendering can be used in, amongst other things, soaps, washing powders, as lipids in the chemical industry and cosmetics (Kalbasi-Ashtari et al., 2008; NABC, 2004). It may also be burnt for energy production and due to its high fat content a considerable amount of energy may be recovered which would otherwise be lost; thus reducing the net environmental footprint of the process (Woodgate & van der Veen, 2004). As with incineration, rendering has a high energy demand but if tallow is recovered for subsequent energy production then the net GHG emissions are likely to be low.

The main environmental concerns associated with rendering relate to gas and odour emissions. Odours may be generated from the raw material, during processing and from the resulting waste effluents (DEFRA, 2008c). Emissions must be prevented, reduced or treated, preferably in that order, using best available technologies (DEFRA, 2008c). In a review of rendering systems, Kalbasi-Ashtari et al. (2008) report that 90% of odours can be removed using cold water washing with further emission reductions achieved using afterburners, scrubbers or biofilters. With regards to effluents generated at rendering plants, suspended solids, oils and greases must be regulated to prevent the release of effluents with high biological and chemical oxygen demand into watercourses. Pollutants can be reduced simply by water use or recycling and reusing, or by treatment on or off-site at conventional sewage treatment works (DEFRA, 2008c).

A hygiene standard of 133 °C /20 min /300 kPa or equivalent is required by the EU for the rendering of high-risk material, including livestock carcasses, to inactivate agents such as

TSEs. As there is no guarantee that the rendering process completely destroys the prions responsible for TSE infections (EC SSC, 1999), SRM must currently be incinerated after rendering (Anon, 2002). Seidel et al. (2006) have shown, however, that alternative strategies to terminal incineration are possible with minimal risk, suggesting that current EU legislation is too constraining, particularly for pork and poultry where there is no evidence of naturally occurring TSEs (EC SSC, 1999). NABC (2004) reports that rendering sufficiently destroys most pathogens but recontamination can occur, particularly with *Salmonella*, during handling, storage and transportation of the final product. However, this can occur with most common municipal and animal waste streams (e.g. compost or digestate) and can be considered to be of low risk if effective handling and storage procedures are in place.

Although the negative issues of biosecurity for carcass collection and transport for rendering are similar to those discussed previously for centralised incineration, it does represent a well established method of livestock disposal for those with access to a central collection service (Tables 1 and 2; Woodgate & van der Veen, 2004). However, commercial rendering facilities are becoming increasingly scarce due to economic pressures on the industry (Anderson, 2002; CAST, 2008b; Kalbasi-Ashtari et al., 2008; Stanford & Sexton, 2006). Traditionally, farmers have been paid to have their livestock mortalities rendered as the revenue from rendering products outweighed the cost of the process, but the inability of the process to completely destroy TSEs has led to the reduction in saleable products, resulting in the introduction of fees (Stanford & Sexton, 2006). Nevertheless, rendering is still a preferred option for disposing of diseased animals in the EU and is likely to continue to be so, preferably in combination with incineration and a pathogen monitoring regime (Anon, 2002; Pollard et al., 2008).

**Table 1.** Grading of the socio-economic and biosecurity aspects of methods used throughout the world for disposal of routine livestock mortalities; assuming best practice.

Method	Socio-economic aspects			Human health	Biosecurity aspects					
	Process speed	Relative cost	Practicality (for the farmer)	Dioxins/ furans	Pathogen contamination of:			Land-spreading of waste produced	Transport of animals off-farm	Prion destruction
					Air (bioaerosols)	Soil and vegetation	Water			
Burial	***	*****	****	*****	****	***	MRN	N/A	*****	*
Burning	****	****	***	**	MRN	MRN	MRN	MRN	*****	***
Incineration (on-farm) <sup>a</sup>	*****	**	***	****	***** <sup>b</sup>	***** <sup>b</sup>	***** <sup>b</sup>	MRN	*****	*****
Incineration (large central facility)	*****	**	*****	***	***** <sup>b</sup>	***** <sup>b</sup>	***** <sup>b</sup>	MRN	*	*****
Rendering	*****	***	*****	MRN	*****	N/A	MRN	N/A	*	****
Composting <sup>c</sup>	**	****	***	MRN	***	***	MRN	MRN	*****	***
Anaerobic digestion	**	*** <sup>d</sup>	***	MRN	****	***	***	MRN	*****	**
Alkaline hydrolysis	****	** <sup>e</sup>	***	MRN	*****	*****	*****	*****	*****	*****

\* Very poor  
 \*\* Poor  
 \*\*\* Moderate  
 \*\*\*\* Good  
 \*\*\*\*\* Very good  
 MRN More research needed  
 N/A Not applicable

<sup>a</sup> Assumes conformation to ABPR (1774/2002) specifications e.g. use of afterburners

<sup>b</sup> Omits handling and storing phase of carcasses pre-incineration which may constitute potential biosecurity risks (Section 2.3)

<sup>c</sup> Assumes unlined static pile with no forced aeration

<sup>d</sup> Benefits from methane production (biogas for energy production) not considered

<sup>e</sup> Unlikely to be suitable for small farms; although increasingly cost-effective with increasing farm size



**Table 2.** Grading of the environmental impacts of methods used throughout the world for disposal of routine livestock mortalities; assuming best practice.

Method	Environmental impacts					
	Odour	Greenhouse gas emission	Pollution and contamination of:			Land-spreading of waste produced
			Air	Soil and vegetation	Water	
Burial	***	****	*****	**	***	N/A
Burning	*	MRN	MRN	MRN	MRN	MRN
Incineration (on-farm) <sup>a</sup>	****	**	**** <sup>b</sup>	**** <sup>b</sup>	**** <sup>b</sup>	MRN
Incineration (large central facility)	*****	**	*** <sup>b</sup>	*** <sup>b</sup>	**** <sup>b</sup>	MRN
Rendering	***	****	MRN	*****	***	MRN
Composting (unlined)	****	****	MRN	***	MRN	****
Anaerobic digestion	****	*****	*****	MRN	MRN	****
Alkaline hydrolysis	***	MRN	MRN	****	***	***

\* Very poor  
 \*\* Poor  
 \*\*\* Moderate  
 \*\*\*\* Good  
 \*\*\*\*\* Very good  
 MRN More research needed  
 N/A Not applicable

<sup>a</sup> Assumes conformation to ABPR (1774/2002) specifications e.g. use of afterburners

<sup>b</sup> Omits handling and storing phase of carcasses pre-incineration which may constitute potential environmental risks (Section 2.3)

## 2.5. Composting

Outside of the EU, aerobic composting is widely used to dispose of livestock mortalities. Composting is a simple technique that can be undertaken on-farm using windrow and bin composting (NABC, 2004), or at dedicated facilities using enclosed windrows or in-vessel techniques (DEFRA, 2008d). Typically, the process involves the layering of carcasses between strata of carbon-rich substrate such as straw, sawdust or rice hulks with a final covering of carbon-rich substrate over the entire pile (NABC, 2004). Larger carcasses are typically placed in single layers while poultry can be multi-layered; and the compost piles are subsequently aerated or turned (NABC, 2004). Depending on carcass weights, the waste material may decompose at rates as high as  $1\text{--}2\text{ kg day}^{-1}$  (Kalbasi et al., 2005) into a useful product that can be used as a soil amendment. The process essentially occurs in two phases – a primary, thermophilic phase (temperatures up to  $70\text{ }^{\circ}\text{C}$  generated for a number of weeks) and a secondary, mesophilic phase (typically  $30\text{--}40\text{ }^{\circ}\text{C}$ ) for a number of months (Kalbasi et al., 2005).

When an impermeable base is not used, small-scale composting of mortalities has been shown to contaminate the underlying soil due to the loss of leachate with a high ionic strength from the compost piles (Glanville et al., 2006); and is likely to be exacerbated under periods of high rainfall. To minimise the risk of pollution (i.e. leaching and runoff), composting should be undertaken on an impervious base (e.g. hard standing or plastic liner) and a bulking agent utilised to absorb excess liquids produced from the decomposing bodies (e.g. sawdust; NABC, 2004). The risk can be further reduced by undertaking the composting indoors or under gas-permeable covers to prevent rain ingress into the compost piles (Sivakumar et al., 2008). This precaution should also prevent run-off and leaching of nutrients as well as reducing ammonia emissions. In terms of gaseous emissions, odour levels from the composting of carcasses are considered to be low in comparison to manure-related facilities (Glanville et al., 2006); and whilst composting carcasses may also lead to GHG emissions, it is unknown whether these emissions are any greater than those released through natural decomposition (Xu et al., 2007).

The temperatures generated during the thermophilic phase of carcass or meat waste composting has been shown to effectively reduce numbers of bacteria, viruses, protozoa and helminths (Glanville et al., 2006; Ligocka and Paluszak, 2008; Wilkinson, 2007). However, some bacteria, particularly *Salmonella*, can re-colonise the compost when temperatures are reduced near the end of the composting process or if the pile has not been adequately aerated

or turned (NABC, 2004; Wilkinson, 2007). It is also possible that opportunistic pathogens may colonise the compost pile if insufficient temperatures are reached (Sanabria-Leon, 2006). In a trial where road-killed deer were composted in a static pile, Schwarz et al. (2008) found that numbers of bacterial indicator species were reduced to near zero after twelve months, but they recommend that a cautious approach be taken and the compost used in areas with limited public contact (e.g. along roads) to further negate any risks. Studies have shown that the avian influenza virus can be deactivated at ambient temperatures (15–20 °C) in less than a week, or after 15 minutes when mixed with chicken manure at 56 °C (Lu et al., 2002); temperatures easily achieved in composting piles. Further, a recent study by Guan et al. (2009) showed that composting rapidly eliminates avian influenza and Newcastle Disease viruses in chicken carcasses. A risk-based review of disposal options for avian influenza by Pollard et al. (2008) placed in-vessel composting on the preferred list of disposal methods on the grounds of exposure assessment. Glanville et al. (2006) showed that a 45 to 60 cm layer of clean material covering cattle carcasses was enough to prevent the compost piles containing vaccine strains of avian encephalomyelitis and Newcastle Disease virus from infecting sentinel birds. When the surface of the compost piles was contaminated with the strains, six out of the twenty two sentinel birds showed positive serum antibody tests, stressing that clean material must be used to cover the composting piles. There is little information regarding the fate of prions or spore forming bacteria such as *Bacillus anthracis* during carcass composting, thus preventing it from becoming considered as an EU-compliant disposal route. However, Huang et al. (2007) found some initially promising evidence in their study with scrapie-infected sheep, with prion removal in one experiment and prion reduction (but not destruction) in the second.

In the foreseeable future, in-vessel composting of routine mortalities, particularly on pig and poultry farms where there is no evidence linking to TSE infection (EC SSC, 1999) could provide a practical, cost-effective and low-risk method of carcass disposal. The use of Geographical Information Systems and Groundwater Vulnerability maps to locate ideal composting sites, along with good composting practices (e.g. using clean and fresh carbon substrate) in tandem with stringent regulation to restrict subsequent land-spreading to specific soil types, a pathogen monitoring regime and a maximum mass of carcasses to be disposed, would further decrease perceived risks. Biosecurity can be improved again by composting in fenced, contained areas (Xu et al., 2009). In summary, although mortality composting is not currently allowed in the EU, there seems to be no scientific evidence to suggest that compost

derived from pig and poultry carcasses should be subject to any greater legislative restrictions than compost derived from municipal food waste.

## 2.6. Anaerobic digestion

Anaerobic digestion (also termed biodigestion) of dead livestock is not permitted within current EU legislation without prior treatment of the carcass, e.g. rendering (Anon, 2002); however the technique is increasingly utilised in other countries. Anaerobic digestion involves the degradation of organic material under anaerobic conditions to produce methane (biogas), which can be utilised as a fuel source (Ward et al., 2008). Other end products include liquid and solid fertilisers (digestate). Digesters can vary in size and technology according to needs and location (Owen et al., 2005). On-farm systems can be as simple as a plastic-covered trench covered with a pipe leading to a storage tank as used in some developing countries (Owen et al., 2005) or large commercial technical plants available for treating large waste volumes (CAST, 2008b). Anaerobic digestion of carcasses can take place at psychrophilic (<20 °C), mesophilic (20 to 45 °C) and thermophilic (45 to 60 °C) temperatures (Cantrell et al., 2008) for different durations. The time–temperature combination affects the physico-chemical conditions within the system and hence the survival of pathogenic agents. Although seemingly one of the most promising technologies to deal with livestock mortalities, biodigestion of carcasses currently remains markedly understudied and most available information relates to the disposal of manure and slurry wastes from farms. However, increasing interest in the disposal of dead livestock is generating research, particularly into the potential of co-digesting of carcasses with other farmyard waste such as manure or slurry. For example, Masse et al. (2008) investigated the addition of ground swine carcasses to swine manure slurry using psychrophilic anaerobic digestion and found no reduction in efficiency.

In the UK, ongoing work seeks to determine the reduction of *Enterococcus faecalis*, *Salmonella senftenberg* and porcine parvovirus in pig carcasses during co-digestion with livestock slurries (Kirby; personal communication). There are some studies on anaerobic digestion of wastewater biosolids and swine manure that have reported varying levels of success at pathogen removal. For instance, Viau and Peccia (2009) found mesophilic anaerobic digestion combined with composting of wastewater biosolids failed to eradicate *Legionella pneumophila* in half of digestate samples. Likewise, Côté et al. (2006) found that

although *Salmonella*, *Cryptosporidium* and *Giardia* were removed during anaerobic digestion, indigenous faecal indicators such as total coliforms had persisted in just over half of samples, although at significantly reduced levels. Nevertheless, there is a plethora of evidence that shows anaerobic digestion can eliminate a range of pathogenic viruses and bacteria from a range of waste matrices (Sahlström et al., 2003, 2008; Viau and Peccia, 2009; Ward et al., 2008). Further, it is also common to include a secondary heat treatment process (e.g. composting or pasteurisation) and a minimum storage period at the end of the process for the digestate as additional measures to inactivate pathogenic organisms (Sahlström, 2003). Grinding waste to smaller particle sizes prior to anaerobic digestion has also been shown to improve sterilisation as it increases the surface area subject to treatment, whilst also increases the rate of subsequent carcass breakdown (Paavola et al., 2006).

TSEs are not destroyed at the operational temperatures of anaerobic digestion (Brown et al., 2000) and have been shown to remain intact through biodigestion of biosolids (Hinckley et al., 2008). Therefore, if infected carcasses are anaerobically digested, digestate potentially contaminated with TSEs can remain in the bottom of the digester (Adkin et al., 2010; Hinckley et al., 2008; NABC, 2004). It is therefore important that techniques are found to remove prions by heat-treating the resulting waste post-digestion as per the EU regulations (Anon, 2002; DEFRA, 2008d). As with composting though, concerns regarding persistence of prions during anaerobic digestion are somewhat irrelevant in terms of pigs and poultry. In environmental terms, anaerobic digestion is evidently the optimal method of carcass disposal as it yields a low-carbon source of power from a waste product. However, if additional treatment of carcasses (e.g. secondary heat treatment) is needed to satisfy biosecurity concerns, this may decrease its environmental credentials.

The initial capital costs, the difficulty in optimising the process in a one-stage reactor and at thermophilic temperatures (Chen and Huang, 2006) may prove to be inhibitory to the uptake of anaerobic digestion as a method of on-farm disposal of livestock mortalities. However, the ability for anaerobic digestion to produce bio-energy makes this an important livestock disposal option given current climate change concerns. Indeed, in the event that existing digesters can be adapted to degrade carcasses mixed with slurry or manure, this method of livestock disposal could prove to be both environmentally sound and economically appealing given the increasing financial incentives for production of bio-energy.

## 2.7. Alkaline hydrolysis

Alkaline hydrolysis was developed in the 1990s and is hence a relatively new technology. It uses sodium hydroxide or potassium hydroxide to catalyse the hydrolysis of biological material (e.g. carcasses) into a sterile aqueous solution consisting of peptides, amino acids, sugars, and soaps (Kaye et al., 1998; NABC, 2004; Shafer et al., 2000, 2001). Carcasses are placed in a steel alloy container to which the alkali is added in either solid or solution form, the concentration of which depends on the weight of the carcass material. The container is then sealed and the process run at 150 °C for up to six hours and at high pressure in order to significantly accelerate the process (EC SSC, 2003b; Kalambura et al., 2008).

Whilst it is reported that there are few gaseous emissions and associated odour problems from alkaline hydrolysis, the effluent is highly alkaline and very rich in nutrients which could pose a problem when discharging the effluent to wastewater treatment systems (NABC, 2004). Indeed, effluent is not currently allowed to be discharged to sewers in the EU without prior treatment so as to prevent the solidification of hydrolysate (EC SSC, 2003b). However, the process has been used with poultry carcasses to produce a fertiliser which can be land-spread (CAST, 2008a). Indeed, recent studies have highlighted the use of the product of alkaline hydrolysis as a highly valuable and effective fertiliser with soil neutralising properties (Gousterova et al., 2008; Kalambura et al., 2008). Alkaline digestion (i.e. alkaline hydrolysis without heating) can also be used as a preservative and the resulting poultry meal has been used as a feed seemingly without detrimental effect (CAST, 2008a); however, in the EU, feeding animals with protein from the same species is prohibited (Anon, 2002).

The combination of high pH (typically *ca.* 14) and a period of sustained elevated pressure and temperature facilitate highly effective eradication of infective agents from carcasses and animal wastes. For instance, both Kaye et al. (1998) and Neyens et al. (2003) showed that alkaline hydrolysis resulted in the near total eradication of pathogenic microorganisms; whilst the former study and more recently Murphy et al. (2009) also proved the effectiveness of alkaline hydrolysis in destroying prions. The EC SSC (2003b) approved this method for the treatment of TSE-infected material provided that the risk of TSE infectivity was excluded from residues. Alkaline hydrolysis is also one of the preferred options of disposal of poultry infected with Avian Influenza H5N1 (Pollard et al., 2008).

Given its effectiveness in eliminating both pathogens and prions from animal by-products, the growth seen in the popularity of alkaline hydrolysis for carcass disposal is of no

surprise. Further, recent papers state that it compares favourably in economic terms to other disposal methods for animal by-products (Gousterova et al., 2008; Kalambura et al., 2008); which is especially true for centralised, large-scale or intensive livestock production systems. It is therefore likely that alkaline hydrolysis will increasingly be at the forefront of methods used to dispose of livestock carcasses both within and outside of the EU.

**Table 3.** The environmental, health and biosecurity aspects of alternative methods for *disposal* of routine\* and large numbers<sup>+</sup> of livestock mortalities.

Method	Environmental and health aspects	Biosecurity aspects	References
<i>Hydrolysis</i> <sup>+</sup> Indirect steam application to a bioreactor where the material is treated at 180°C/40°/12 bar.	Produces a biofuel.	Not deemed suitable for TSE-infected material.	EC SSC (2003a) Cantrell et al. (2008)
<i>Gasification</i> <sup>+</sup> Uses high temperature combustion in excess oxygen to oxidise organic matter.	Production of NO <sub>2</sub> , SO <sub>2</sub> & CO gases, VOCs, PAHs, dioxins and furans and particulate matter including ash. Less air emissions released than standard incineration.	Not deemed suitable for TSE-infected material. Preferred option in the disposal of Avian Influenza Virus H5N1.	Hetland and Lynam (2001) EC SSC (2003a) Cantrell et al. (2008) Pollard et al. (2008) CAST (2008a)
<i>Thermal depolymerisation</i> <sup>+</sup> Uses high heat and pressure to convert organic matter into a biofuel.	Produces re-useable combustible gas and a biofuel. Waste minerals to be used as fertiliser.	Expected to destroy prions and pathogens as the process destroys organic matter at the molecular level. Carcasses pre-processed on-farm and transported in sealed containers, improving biosecurity.	NABC (2004)
<i>Plasma arc process</i> <sup>+</sup> High heat torch used to vitrify or gasify material into a reduced volume solid.	Remaining solids can be land-filled or used as gravel, moulded into bricks or used as concrete aggregate. Methane produced contributes to global warming if not captured.	Expected to destroy prions and pathogens. Carcasses pre-processed on-farm and transported in sealed containers, improving biosecurity.	Hetland and Lynam (2001) NABC (2004)
<i>Ocean disposal</i> <sup>*+</sup> Dumping of carcasses beyond territorial limits.	Additional nutrient loading at dumping sites. Would need to prevent floating debris. More research needed.	Potential spread of parasites and pathogens, although likely to be diluted and have limited survival.	NABC (2004)
<i>Napalm</i> <sup>*+</sup> Use of fast-burning napalm to replace burning pyres.	Burning would produce emissions to air, ash and contamination of soil and groundwater. Health issue when using and handling napalm.	Expected to destroy pathogens although no conclusive information currently available.	NABC (2004)



**Table 3.** Continued...

Method	Environmental and health aspects	Biosecurity aspects	References
<i>Pyrolysis</i> <sup>+</sup> Use of electromagnetic waves to heat organic material – not yet tested on carcasses.	Reported to reduce emissions and hydrocarbons; low energy requirements. Only small amounts of waste produced.	Expected to destroy TSEs and pathogens, although no conclusive information currently available.	Hetland and Lynum (2001) NABC (2004) Cantrell et al. (2008)
<i>Natural exposure</i> <sup>*</sup> Use of natural processes and predators to remove carcasses.	Many potential environmental and health implications. Only an option in scarcely populated areas.	Potential spread of parasites and pathogens.	Anon (2007) Stanford and Sexton (2006)
<i>Extrusion</i> <sup>+</sup> Use of friction to grind and ‘cook’ poultry carcasses. Moisture removal and the addition of a dry ingredient turns waste carcass into feed.	Unknown. Possibly harmful if process is unregulated and contaminated feed is fed to livestock animals.	No information on TSEs; though elimination of pathogens. Possibly harmful if process is unregulated and contaminated feed is fed to livestock animals.	Blake (2004)

**Table 4.** The environmental, health and biosecurity aspects of alternative methods for storage of both routine\* and large numbers<sup>+</sup> of livestock mortalities.

Method	Environmental and health aspects	Biosecurity aspects	References
<i>Bioreduction</i> * Carcasses stored in a vessel containing water, where the contents are heated and aerated. Used for volume reduction prior to disposal	Stored in watertight containers therefore no environmental impact from leakage or seepage expected. GHG emissions being investigated.	Reduced number of on-farm collections. Bioaerosol generation and pathogen survival being investigated.	Williams et al. (2009)
<i>Freezing</i> *+ Storage of carcasses on-farm and transported in a refrigerated unit in larger quantities.	Stored in sealed containers so little environmental impact. Energy consumption needs to be balanced against transport savings made.	Pathogen eradication unlikely; however carcasses can be stored in sealed units to reduce chance of propagation.	NABC (2004) Blake (2004) CAST (2008a)
<i>Lactic acid fermentation</i> <sup>+</sup> 'Pickling' of animal carcasses when inoculated with <i>Lactobacillus acidophilus</i> and a carbon source in an anaerobic environment at ~30°C. Carcasses must be ground first.	Fermentation may not complete if putrefaction is allowed to start before carcasses are fermented. If the rendered material is turned into feed then it may contain toxic amines. Process is sealed so little environmental threat expected.	Low pH (optimum 4.5) and heat treatment (~30°C) should deactivate most pathogens. Rendering should complete the process. No information on TSE persistence.	NABC (2004) Blake (2004) CAST (2008a)
<i>Grinding &amp; storing</i> *+ Grinding of carcasses and storage in chemicals (e.g. inorganic acid) or heat-treatment in sealed units.	Storage in sealed containers should have little environmental impact unless preservative is spilt <sup>a</sup> .	Grinding speeds up decomposition therefore waste needs quick disposal, unless preserved. Grinding may improve subsequent eradication of pathogens; however may constitute a risk at times of disease outbreaks (e.g. avian influenza).	Lo et al. (1993) NABC (2004) CAST (2008a) CAST (2008b) Cai et al. (1995)
<i>Yeast fermentation</i> <sup>+</sup> Similar to lactic acid fermentation. Ground carcasses added to an agitated tank with a Carbon source and yeast inoculant. Kept at ca. 26-29°C.	Unknown.	Some pathogens shown to recover 12 h and 48 h post-inoculation.	Blake (2004)

<sup>a</sup> Author's opinion

### **3. The future of livestock mortality disposal**

#### *3.1. Novel disposal methods*

Novel methods of livestock disposal are briefly summarised in Table 3. These have not been discussed thoroughly in the text as they are currently unlikely to be economically viable for most farmers or considered to be environmentally safe and biosecure for the foreseeable future. Further work will be needed on these aspects if they are to be developed and utilised on a commercial scale and more importantly if they are to gain legislative acceptance.

#### *3.2. Carcass storage and bioreduction methods*

In addition to the different methods of carcass disposal, there are several potential options that allow carcasses to be stored safely on-site prior to disposal via one of the approved routes previously discussed. The main advantage of storing carcasses is that farmers can wait until it is economically viable and convenient to organise their disposal, and in some cases the volume of livestock can be decreased therefore reducing disposal costs. A summary of storage methods is provided (Table 4), although the two most likely to be appealing and practical for farmers, bioreduction and freezer storage, are discussed briefly here.

Bioreduction is a method which simultaneously permits storage and reduction in the volume of carcasses and relies on internal enteric microorganisms and enzymes to drive decomposition. Briefly, carcass material is placed in a watertight vessel, where the contents are heated (to  $40 \pm 2$  °C) and actively aerated with a pump. In contrast to in-vessel composting or anaerobic digestion, the process relies on an aqueous environment to promote microbial degradation of organic material. To facilitate this, vessels are two thirds filled with water prior to carcass addition. During storage, the putrescible carcass material liquefies, facilitating liquid phase disposal; and a reduction in volume occurs due to evaporation through an air vent (Williams et al., 2009). Heating encourages microbial replication, whilst regular aeration facilitates eradication of zoonotic gut pathogens due to them predominantly being facultative anaerobes. Work on bioreduction so far has focussed on sheep mortalities, but anaerobic bioreduction has been studied on pig and rabbit farms in Spain (Gutiérrez et al., 2003; Lobera et al., 2007a, 2007b). It is analogous to aerobic bioreduction but without a direct input of air, but differs to anaerobic digestion as the system is not fully sealed since the aim is not to produce (or capture) methane for bio-energy production. Although the

technology is in its infancy and has not yet been studied with larger carcasses such as cattle or horses, early results for bioreduction are promising. Both the aerobic and anaerobic bioreduction systems have been shown to be highly effective with regards to the rate of carcass breakdown (Lobera et al., 2007a, 2007b; Williams et al., 2009). Once full, the liquid portion of the vessels is emptied via vacuum suction and is subsequently incinerated or rendered. However, as the volume of waste is considerably reduced, it must only be disposed of intermittently; which may reduce the environmental footprint associated with carcass disposal and also alleviate biosecurity concerns associated with collecting vehicles frequently accessing different livestock holdings (Kirby et al., 2010; Williams et al., 2009).

Bioreduction may cause some biosecurity concern, especially in the form of bioaerosols due to the active aeration of the contents. However, both aerobic and anaerobic bioreduction systems appear to reduce survival of enteric bacteria potentially present in livestock; including *Salmonellae*, *E. coli* and *E. coli* O157 (Gutiérrez et al., 2003; Williams et al., 2009), *Clostridium* (Lobera et al., 2007a, 2007b) and *Campylobacter* (Williams et al., 2009). Further work is needed to determine the survival of bacterial pathogens and viruses when the bioreduction system is not managed under optimal conditions (e.g. when the air and heat input is switched off).

The potential for TSEs to persist within a bioreduction system and the risk of subsequent propagation was recently evaluated in a systematic review (Adkin et al., 2010). It was concluded that microbial processes and enzymatic breakdown of proteins (proteolysis) was likely to lead to the degradation of TSEs. However, prions have been shown to be resistant to proteases and the mesophilic temperatures within the vessels are not sufficiently high to deactivate the protein (Brown et al., 2000). As a result, it is possible that a proportion of prions would adhere to the solid component of the waste material and settle to the bottom of the vessel, where they could remain in a potentially infective state (Adkin et al., 2010). Nevertheless, the assessment concluded that the risk of TSE agents being dissipated through the chimney via gaseous emissions were likely be negligible (a 1 in  $1 \times 10^{12}$  probability over a one year period), and their exit via aerosols through the opening hatch during operational procedures was only of slightly greater concern (Adkin et al., 2010). The findings of the review by Adkin et al. (2010) will soon be validated through *in-vitro* models to deduce the fate of prions in bioreduction systems in order to better inform future risk assessments.

In the event that prion and pathogen destruction within the liquor is proved, it is possible that alternative methods of disposal can be utilised for the liquor such as treatment

via lime stabilisation (Avery et al., 2009) or co-composting followed by land-spreading in suitable areas. This may reduce biosecurity fears due to the containment of the entire process on-farm and would also include the added benefit of closing the nutrient cycle. Since there is no evidence linking either poultry or pigs to TSE infection (EC SSC, 1999), the resulting waste from bioreduction of such carcasses may certainly be suitable for land-spreading if further work substantiates that the liquid waste produced poses limited biosecurity and environmental threat. If mismanaged (e.g. if anaerobic conditions are allowed to develop), odour can be an issue of concern during bioreduction (Williams et al., 2009). However, ongoing trials have shown that odour may be alleviated through the use of a woodchip biofilter (Williams et al. unpublished). Future studies are needed to elucidate the temporal changes in microbial communities during bioreduction and optimisation of enzymatic degradation processes in order to improve the process and facilitate legislative approval.

Freezing of mortalities retards the rate of decomposition by lowering the core temperature of the carcasses (NABC, 2004). Depending on the volumes of mortalities, facilities can be as simple as using chest freezers or loading carcasses into cold storage until disposal is required (NABC, 2004). As with bioreduction, its appeal arises due to the reduced frequency for off-farm transportation of small volumes of carcasses and hence improved levels of biosecurity. In contrast to bioreduction however, the volume of waste does not decrease during freezer storage and therefore it is only likely to be suitable for farms that generate small quantities of mortalities (e.g. <50 kg per day (Blake, 2004)). Freezing is probably most applicable to poultry (Blake, 2004) and pig (CAST, 2008b) enterprises; however, it has also been used effectively to store larger species as a contingency prior to disposal during disease outbreaks such as FMD and BSE (de Klerk, 2002; NABC, 2004). Nevertheless, little is mentioned in the literature regarding on-farm freezing of carcasses and animal by-products which probably relates to the potential for considerable running costs, and the ABPR (1774/2002) only mentions it in the context of Category 3 intermediate plants that may temporarily store animal by-products by freezing into blocks prior to disposal.

The cold storage of carcasses is not meant to destroy pathogens and infective agents but rather to prevent their proliferation and reduce further carcass decay whilst storing for bulk disposal (CAST, 2008b). Prions are known to remain viable after freezing for considerable lengths of time (Stamp, 1967). Zoonotic pathogens such as *Campylobacter* (Maziero and de Oliveira, 2010; Sandberg et al., 2005), *Salmonella* (Escartin et al., 2000) and *E. coli* O157 (Dykes, 2000) have been detected in frozen raw meat, whilst *Cryptosporidium* have been

isolated from cattle faeces after periods of freezing (Olson et al., 1999). However, all studies reported a significant decrease in numbers of these organisms following the freezing period. Indeed, freezing is used as a pre-treatment method for reducing *Campylobacter* sp. in broiler chickens (Georgsson et al., 2006, Loretz et al., 2010, Rosenquist et al., 2009). For non-ruminant carcasses where TSEs are not of concern, freezer storage prior to ultimate disposal may therefore actually yield unexplored benefits in terms of biosecurity.

Environmental costs are inevitable when a constant use of electricity is required, as there is for freezing. However, energy-efficient freezers are increasingly available and the potential GHG savings made by reducing the transport of carcasses may compensate for this energy expenditure. As with bio-reduction, a detailed life-cycle assessment for a number of case-study farm scenarios is needed to identify the potential cost-benefits to the environment. Another environmental factor related to freezing is the potential for spills to occur when loading carcasses into cold storage containers (NABC, 2004). Effective handling areas and the ability to sanitize such facilities must therefore be implemented if freezing is to be a successful on-farm method of pre-disposal storage.

#### **4. Conclusions**

There are many disposal options for dead livestock currently in use throughout the world; however, the knowledge that TSEs and some pathogens may not be completely destroyed may limit their utility in the wake of changing legislation (e.g. the amended EU Animal By-Products Regulation (1069/2009) which comes into effect in March 2011). On-farm disposal methods are favoured by the farming community due to the perceived environmental, practical, economical and biosecurity benefits, therefore processes such as composting and anaerobic digestion have found favour in countries such as the USA and Canada. Under the ABPR in the EU, these options are not deemed safe; however, the legal alternatives are not favoured by the farming community leading to widespread non-compliance and potentially greater environmental risk (due to illegal dumping, etc. (Kirby et al., 2010)). There is therefore a real need for new methods to be developed and validated and the legislation reconsidered following submission of new evidence. From this perspective, bio-reduction and freezing seems to be promising on-farm storage methods for livestock mortalities, limiting the need for off-farm transport thus reducing associated biosecurity risks.

While the implementation of highly precautionary, risk-averse mortality disposal systems is admirable in many ways, similar risk assessments and legislation do not apply to other components of the livestock sector which may pose a similar or even greater risk to human health or environmental contamination (e.g. spreading of animal waste, animal access to watercourses, public access to grazing land). It is important therefore that mortality disposal systems are based on a realistic and proportionate level of acceptable risk in comparison to other components of the food chain, rather than the current zero-risk approach. It is clear that more evidence is needed on each disposal and storage method in order to make substantiated risk assessments, e.g. the effects of spreading carcass ash on crops or the potential of leachate from burial to contaminate ground or surface water. This review has initiated this process by applying a simple five-star award system to each livestock disposal and storage method (Table 3 and Table 4, respectively) in order to rudimentarily classify various biosecurity and environmental factors based on current scientific evidence. Methods in need of greater research have also been highlighted where there is either limited or no existing published literature. Further research into the economic impacts of dead livestock disposal is necessary for legislators to appreciate the cost implications on the livestock sector, whilst life-cycle assessments are needed to help provide more environmentally sustainable disposal solutions.

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## **Fate of pathogens in a simulated bioreduction system for livestock carcasses**

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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.*

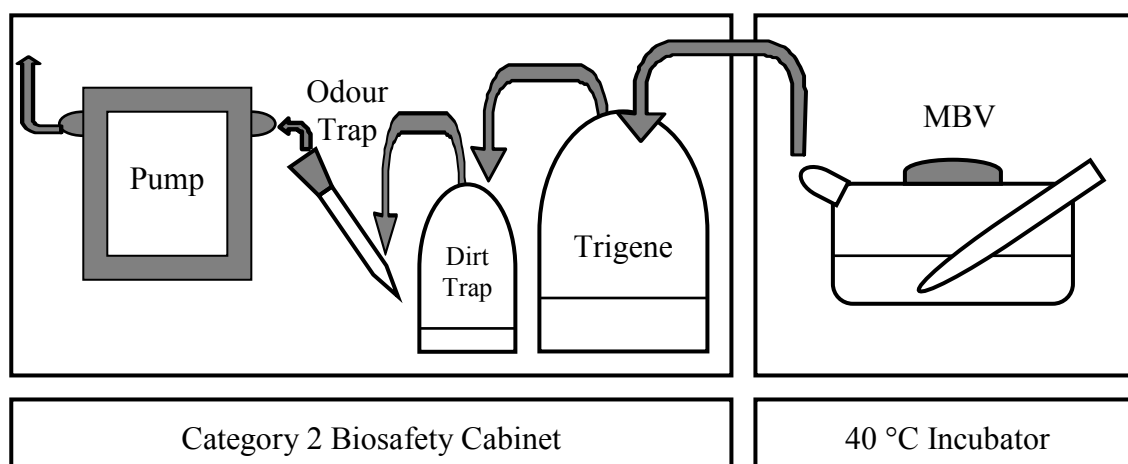
Senftenberg) and *Enterococcus faecalis* (Bohm, 2008). A previous field-scale study on bioreduction of sheep recovered negligible numbers of pathogens (Williams et al., 2009), but the initial pathogen concentration was not high enough to validate whether or not a 5-log reduction in numbers had occurred. 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 ( $\pm 2$  °C) and the contents continuously aerated at a maximum rate of 6 l min<sup>-1</sup>. 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).



**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.

## 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  $231 \pm 1.5$  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<sup>®</sup>, 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 Tn5 *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<sup>-1</sup>) 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<sub>10</sub> CFU of *Salmonella*, 7.89 log<sub>10</sub> CFU of *E. faecalis*, 7.5 log<sub>10</sub> CFU of *E. coli* O157 and 6.81 log<sub>10</sub> 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<sup>-1</sup> 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<sup>-1</sup>) 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 bioeducer by silicone tubing and activated for 30 min at a flow rate of 10 l min<sup>-1</sup>. 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<sub>10</sub> (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

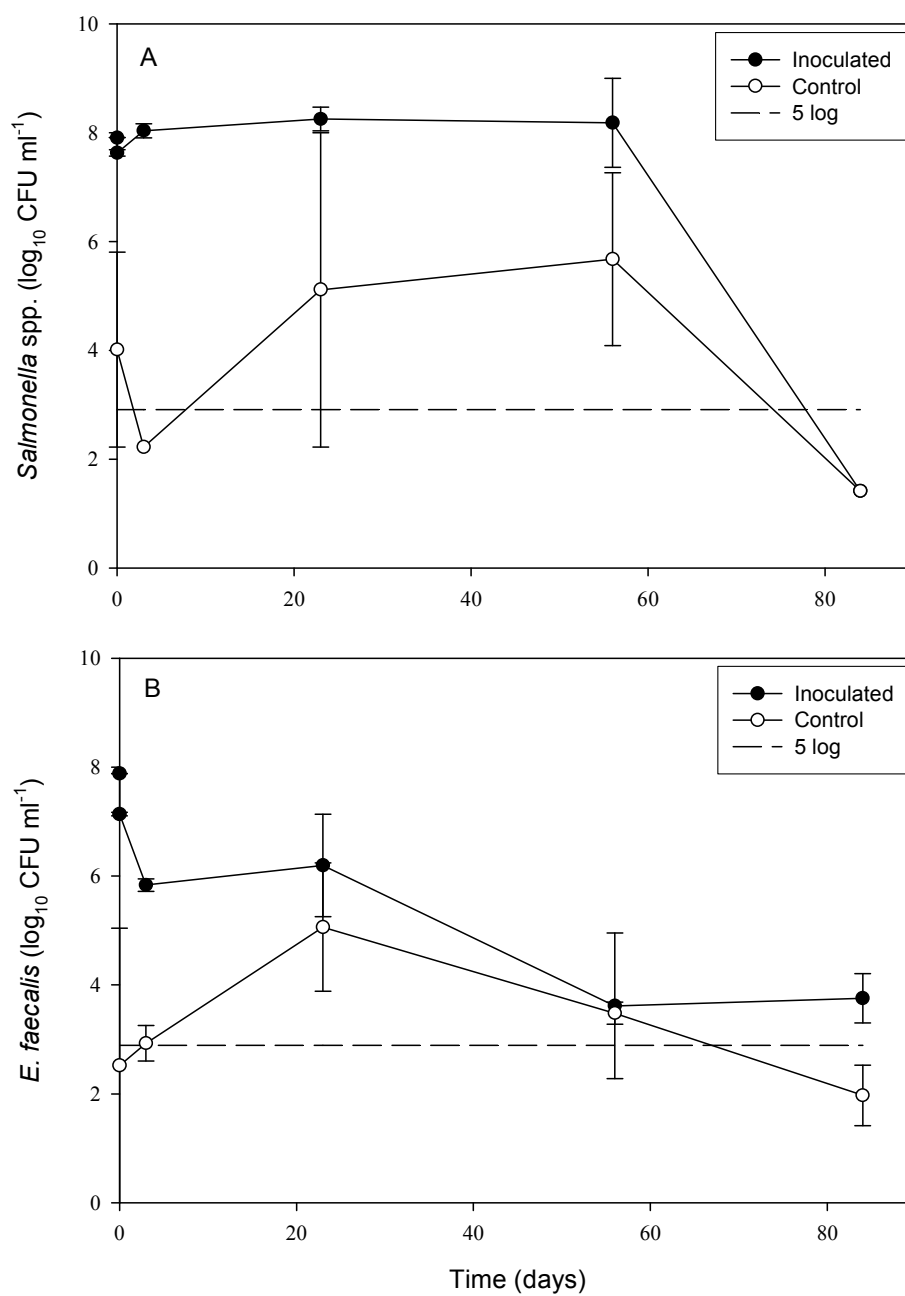
#### 3.1. Waste degradation

At the end of the trial, the reduction in volume of carcass components in each vessel was similar ( $88.2 \pm 3.7\%$  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.

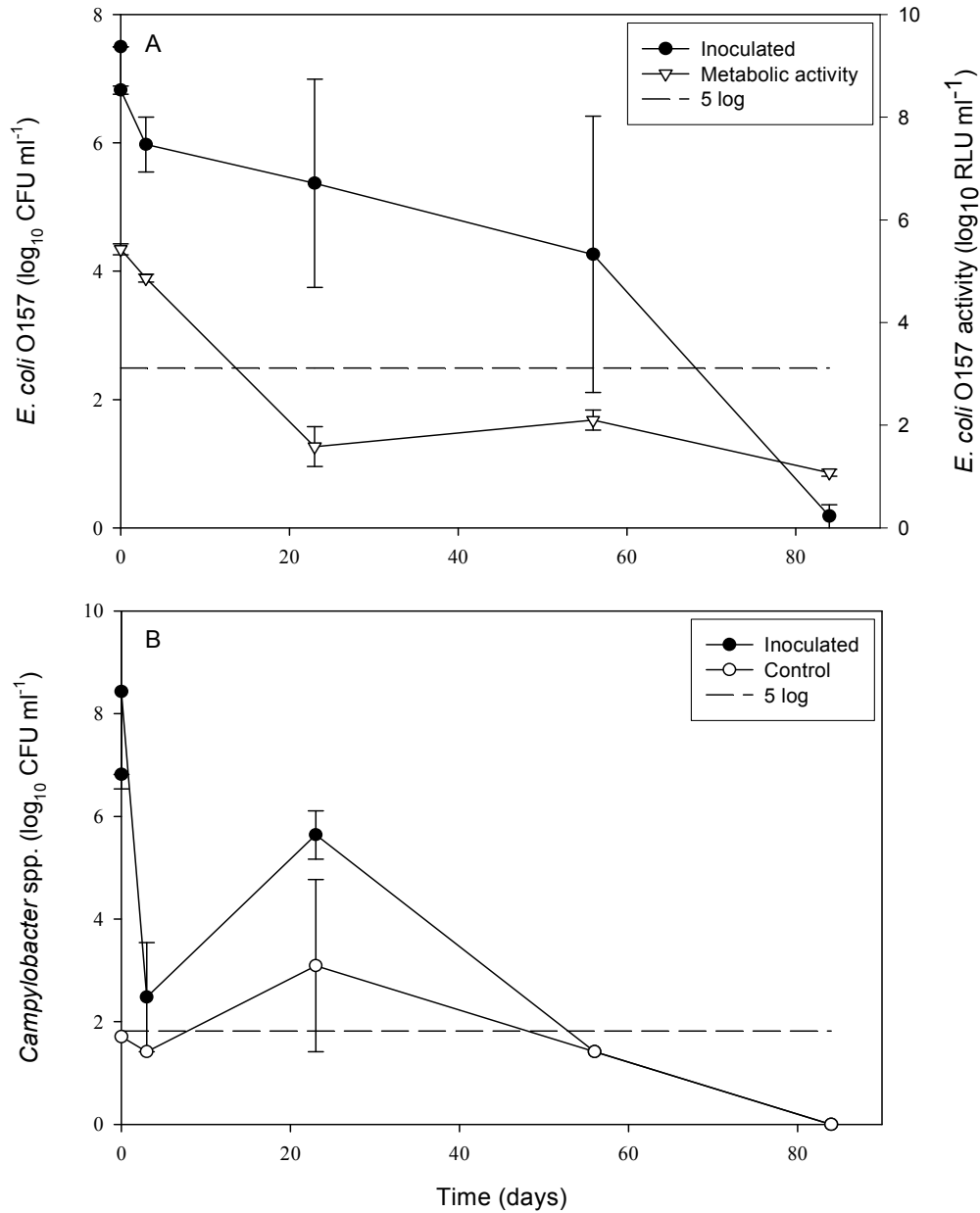
#### 3.2. Microbiological characteristics

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) ( $P > 0.05$ ).

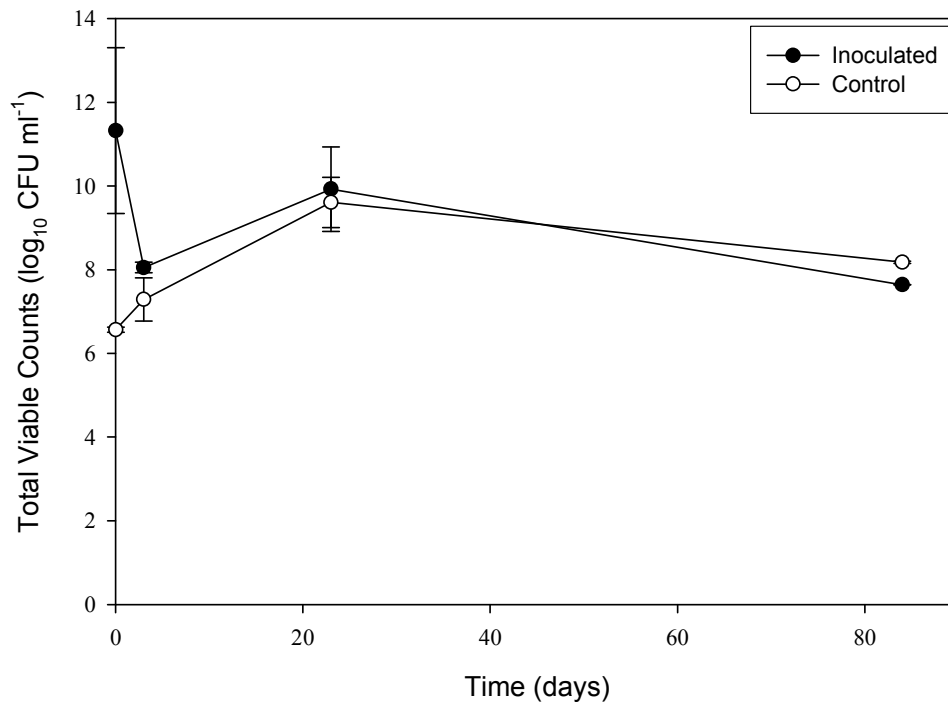
A significant ( $P < 0.05$ ) decline in both the numbers and activity of *E. coli* O157 were seen in the inoculated MBVs (Fig. 3A), culminating in a 5-log reduction by day 84 and luminescence values falling to below background levels. *Campylobacter* spp. numbers declined significantly ( $P < 0.05$ ) 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.



**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  $\pm$  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  $\pm$  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  $\pm$  standard error of the mean.

### 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.

**Table 1:** Detection of bioaerosols from the inoculated mini bioreduction vessels (CFU m<sup>-3</sup>). Values represent means  $\pm$  standard error of the mean. ND denotes not detected.

Day	<i>Salmonella</i> spp.	<i>E. faecalis</i>	<i>E. coli</i> O157	<i>Campylobacter</i> spp.	Total Viable Counts
0	31.9 $\pm$ 21.2	15.6 $\pm$ 10.9	ND	ND	15.7 $\pm$ 6.3
24	ND	4.4 $\pm$ 4.4	ND	ND	147.8 $\pm$ 126.7
57	ND	1.1 $\pm$ 1.1	ND	ND	2925.6 $\pm$ 2917.2
85	ND	ND	ND	ND	41.3 $\pm$ 31.3

#### 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<sup>®</sup> (Biopolym, Spain), was used in this trial. Gel-60<sup>®</sup> 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<sup>®</sup> 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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**Bioreduction of sheep carcasses effectively contains and reduces pathogen levels under operational and simulated breakdown conditions**

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## ABSTRACT

Options for the storage and disposal of animal carcasses are extremely limited in the EU after the introduction of the EU Animal By-Products Regulations (EC/1774/2002), leading to animosity within the livestock sector and the call for alternative methods to be validated. Novel storage technologies such as bioreduction may be approved under the ABPR provided that they can be shown to prevent pathogen proliferation. We studied the survival of *Enterococcus faecalis*, *Salmonella* spp., *E. coli* O157 and porcine parvovirus in bioreduction vessels containing sheep carcasses for approximately 4 months. The vessels were operated under two different scenarios: (A) where the water within was aerated and heated to 40 °C, and (B) with no aeration or heating, to simulate vessel failure. Comprehensive microbial analysis verified that pathogens were contained within the bioreduction vessel and indeed reduced in numbers with time under both scenarios. This study shows that bioreduction can provide an effective and safe on-farm storage system for livestock carcasses prior to ultimate disposal. We propose that these findings support previous work which should lead to a change in the regulatory framework and that bioreduction is approved for industry use within the EU.



**Keywords:** agriculture; animal slaughterhouse waste; fallen stock; mortalities; zoonoses.

## 1. Introduction

The implementation of the Animal By-Products Regulations (ABPR, EC No. 1774/2002) prohibited the burial and burning of livestock carcasses in the EU (Anon, 2009). However, due to concerns by industry about the costs, practicality and biosecurity of the centralised collection system that was implemented for livestock mortalities, unsanctioned disposal of carcasses is known to occur (Gwyther et al., 2011; Kirby et al., 2011). Novel methods of disposal of livestock carcasses and animal by-products may be proposed under the current ABPR provided that the method is proven to be biosecure; that is, lead to a 5-log reduction in numbers of indicator bacterial pathogens (*Enterococcus faecalis* and *Salmonella enterica* serovar Senftenberg) and a 3-log reduction in numbers of indicator viruses (parvovirus) (Bohm, 2008).

Bioreduction has been proposed as a practical and effective interim method for storing fallen stock (livestock carcasses) whilst actively reducing the final volume to be disposed (Williams et al., 2009). The process has been described in detail previously (Gwyther et al., 2011; Williams et al., 2009) and essentially entails the aeration and mesophilic heating of carcasses in a partially sealed vessel containing water. The conditions within the vessels enhance the biodegradation of carcasses by internal microorganisms and enzymes, hence reducing the volume of waste (Williams et al., 2009). The resulting waste is ultimately disposed of by the permitted route for Category 1 material under the ABPR, i.e. via rendering or incineration (Anon, 2009). Although there is significant interest in this new technology by industry, it is currently not authorised for use due to insufficient scientific evidence about its biosecurity attributes. EU Regulations 1069/2009 introduces the concept that animal by-products may be contained on-farm prior to disposal in a manner which controls the proliferation of pathogens (Anon, 2009). By adopting the stringent biosecurity criteria used to ratify carcass disposal methods (described previously) for carcass *storage* methods *prior to* disposal, this should signify whether bioreduction could be used by industry as a way of safely storing fallen stock prior to disposal off-farm.

Previous studies using ovine carcass constituents in simulated bioreduction vessels under laboratory conditions demonstrated that after three months, the numbers of introduced pathogens such as *Salmonella* spp. (including the *enterica* serovar, Senftenberg), *Campylobacter* spp. and *E. coli* O157 decreased by 5-log values, and *Enterococcus faecalis* by 4-logs (Gwyther et al., 2012). Furthermore, only minimal numbers of these organisms were isolated in bioaerosols, and only during the initial stages of the trial just following



inoculation (Gwyther et al., 2012). Nevertheless, further work is needed to validate these findings at the field-scale. The aim of this work was to monitor the fate of introduced bacterial and viral pathogens within bioreduction vessels containing sheep carcasses over a period of four months, under both optimal/operational (regular heating and aeration) and sub-optimal (no heating or aeration) conditions.

## **2. Materials and methods**

### *2.1. Bioreduction vessel design and location*

Three 6,500 l capacity bioreduction vessels (BVs) made of high-density polymer and thermostable glass fibre were commissioned at Bangor University's Henfaes Research Station, UK (53°14'05"N, 40°00'50"W). The vessels were slightly different in construction to those previously described by Williams et al. (2009) being horizontally-, rather than vertically-orientated (3 m diameter  $\times$  2.5 m high). However, they were similar in all other respects. The vertically-orientated vessels were used in this study as controls ( $n = 2$ ) and were not inoculated with pathogens. All vessels were filled with 1,000 l of water prior to the addition of carcasses, heated to  $40 \pm 2$  °C and aerated at a rate of  $117 \text{ l min}^{-1}$  for  $45 \text{ min h}^{-1}$ . The five vessels were connected to a central pipe leading to a biofilter bed of approximately  $1 \times 2 \times 3 \text{ m}$  dimensions (h  $\times$  w  $\times$  d), comprising of compost and woodchip (approximately 20:80 by volume), which was used to reduce odours emanating from the vessels. A commercial catalyst, Gel-60<sup>®</sup> (Biopolym, Spain) containing sodium alginate as the active ingredient, was added to the vessels with each carcass loading according to manufacturer's instructions (1 g Gel-60<sup>®</sup> to 1 kg carcass) in order to maintain consistency with previous bioreduction trials (Lobera et al., 2005; Williams et al., 2009).

### *2.2. Trial management*

Trial A (vessels managed under optimal/operational conditions) ran from January – May 2010. Prior to the experiment, a single sheep carcass was placed into each of the BVs to 'seed' the vessels, and left for a month. Sheep carcasses were subsequently added to each of the five vessels in a single loading of up to 206 kg and a further 2,000 l of water was added (Table 1). An incision was made to the abdomen of each animal without piercing the gastrointestinal system before inputting into the vessels (Williams et al., 2009). Further carcass additions of up to 269 kg per vessel were added equally between the vessels as and

when sheep died on-farm. The water level was monitored visually to ensure that two-thirds of each carcass (by height) was covered at all times (Gutiérrez et al., 2003; Lobera et al., 2007). At the end of Trial A, the waste was disposed of via suction under vacuum, followed by incineration. Clean water was then placed in the vessels to which commercial strength hydrogen peroxide was added for sterilisation.

**Table 1:** Weight of sheep carcasses added to each vessel during Trial A and the time of additions. BV = bioreduction vessels; CONBV = control bioreduction vessels.

Vessel no.	Carcass starting weight (kg)	Carcass additions (kg)	Total weight added (kg)	Time of carcass additions
BV1	151	199	350	Day 29, 68, 86, 92
BV2	130	217	347	Day 16, 29
BV3	206	139	345	Day 29, 66, 92
CONBV1	0	269	269	Day 21, 66, 92
CONBV2	150	176	326	Day 0, 66, 86

Trial B (vessels managed under non-optimal or simulated breakdown) conditions) ran from September, 2010 – January, 2011. Vessels were prepared as described for Trial A, being ‘seeded’ with a single sheep carcass one month prior to starting the experiment. At the beginning of Trial B, 300 kg of fresh carcasses were added in one loading and all vessels (treatment and controls) switched off for the duration of the trial.

### 2.3. Bacterial and viral strains

*Salmonella enterica* serovar Typhimurium (strain S510720, hereafter referred to as *S. Typhimurium*) isolated from sheep, was kindly donated by Mairiead MacLennan of the Scottish Agricultural College. *Salmonella enterica* serovar Poona (NCTC4840, hereafter referred to as *S. Poona*) and *Enterococcus faecalis* (ATCC 29212) were obtained from Oxoid (Oxoid Ltd., UK) and *Salmonella enterica* serovar Senftenberg (NCTC13385, hereafter referred to as *S. Senftenberg*) from HPA culture collections (Health Protection Agency, UK). A *lux*-marked strain of *E. coli* O157 and an environmental strain of *E. coli* O157 (#3704) were used to represent verocytotoxic *E. coli* (Bolton et al., 1999; Kudva et al., 1998; Ritchie

et al., 2003). All media were bought from Oxoid. Porcine parvovirus (PPV) originally isolated from infected swine in Canada was provided by the Agri-Food and Biosciences Institute, UK (Allan et al., 2000).

#### 2.4. Inoculation of vessels

The three treatment vessels were inoculated on day 0 (day of main carcass loading) for both trials. All bacterial pathogens were prepared equally for both trials, although in Trial B only *S. Senftenberg*, *E. faecalis* and *E. coli* O157 (*lux*) were used. Overnight cultures of bacteria from frozen glycerol stocks were grown in Tryptone Soya Broth (TSB; CM0129) at 37 °C with shaking at 150 rev min<sup>-1</sup>. Strains of the same organism were combined into fresh TSB and single strain cultures were sub-cultured again into fresh TSB. A total of 3,000 ml of inoculum was added to each vessel for each bacteria assayed after a final 18 hours of incubation. Background counts from the liquor in the vessel prior to inoculation, and the inoculums added were analysed by direct plating. Starting counts were taken as the sum of both background and inoculated bacteria and amended according to the volume of water and weight of carcasses added, to give a final concentration per ml of vessel contents. Final concentrations per ml of liquid in each treatment vessels were 6.35 log<sub>10</sub> CFU ml<sup>-1</sup> of *E. coli* O157, 5.0 log<sub>10</sub> CFU ml<sup>-1</sup> of *Salmonella* spp., and 5.47 log<sub>10</sub> CFU ml<sup>-1</sup> of *E. faecalis* for Trial A; and 5.06 log<sub>10</sub> CFU ml<sup>-1</sup> of *E. coli* O157 (*lux*), 5.22 log<sub>10</sub> CFU ml<sup>-1</sup> of *S. Senftenberg* and 5.36 log<sub>10</sub> CFU ml<sup>-1</sup> of *E. faecalis* for Trial B.

The virus pool was grown by inoculation of stock virus into T125 flasks containing a semi-confluent monolayer of primary pig kidney cells. Cells were monitored for cytopathic effect (CPE) and flasks harvested when the majority of cells had collapsed. The contents of all the flasks were pooled together and frozen and thawed three times to lyse any remaining cells. The pool was then stored at -80 °C until required. Frozen stocks of PPV were defrosted and were added to the vessels in Trial A at day 29 with the addition of a fresh contingent of carcasses. For Trial B, PPV addition occurred on day 0 along with the bacterial pathogens and addition of carcasses. Final infectious titres of PPV per ml of liquid in each treatment vessel equated to 5.02 log<sub>10</sub> TCID<sub>50</sub> ml<sup>-1</sup> for Trial A and 4.00 log<sub>10</sub> TCID<sub>50</sub> ml<sup>-1</sup> for Trial B and was based on the titre of the PPV inoculum (9.00 log<sub>10</sub> TCID<sub>50</sub> ml<sup>-1</sup> and 8.00 log<sub>10</sub> TCID<sub>50</sub> ml<sup>-1</sup> for Trial A and Trial B respectively) and the volume of water and weight of carcasses added to the vessels..

## 2.5. Sample collection

Liquor samples ( $n = 3$ , 500 ml) were collected from each vessel at a depth of 1 m below the surface of the liquor on days 0, 0.04 (1 hour), 7, and 28 post-inoculation, and once a month thereafter until the end of the trial at day 127. Triplicate biofilter samples ( $n = 3$ , 500 g) were taken from the top 10 cm of compost/woodchip. Both biofilter and bioaerosol samples (see section 2.7) were collected up to 48 hours after each liquor sampling date. Samples were refrigerated and analysed within a maximum of 24 h on return to the lab. Sub-samples were frozen at -80 °C for viral isolation and quantitative PCR, and in glycerol for bacterial DNA analysis.

## 2.6. Liquor waste

### 2.6.1. Bacterial analysis

#### 2.6.1.1. Enumeration of bacteria from liquor and biofilter

Liquor samples were analysed for background counts of *E. coli* O157, *Salmonella* spp., *E. faecalis*, and Total Viable Counts (TVC) according to Gwyther et al. (2012) prior to the addition of inoculums. Biofilter samples (25 g) were shaken at 200 rev min<sup>-1</sup> for 15 min with maximum recovery diluent (MRD; CM0733) (225 ml) prior to analysis for background bacterial counts as for liquor samples. *Salmonella* spp. were enumerated using both Xylose-Lysine-Desoxycholate Agar (XLD; CM0469) and Brilliant Green Agar (BGA; CM0263) during Trial A to try and reduce the number of false positives due to the high microbial background within the matrix (Malorny et al., 2008). The BGA method was replaced in Trial B with an improved XLD method where an additional supplement was added to the media (novobiocin supplement; SR0181); again to limit contamination of the selective media with background microflora (Elving et al., 2010; Restaino et al., 1982).

All presumptive colonies were sub-cultured onto nutrient agar and incubated at 37 °C for 24 hours. Colonies were confirmed using a variety of relevant tests described previously (Gwyther et al., 2012) including latex agglutination and biochemical tests, where appropriate.

#### 2.6.1.2. Bacterial enrichment

Where no *Salmonella* spp. or *E. coli* O157 were isolated, samples were enriched to detect low viable counts. *Salmonella* enrichment was based on ISO standard 6579:2002

(FSA, 2009) using both Rappaport Vassiliadis broth (CM0669) and Muller Kauffmann tetrathionate-novobiocin broth (MKTTn; CM1048, containing iodine-iodide solution and novobiocin supplement, SR0181) as enrichment media. In trial B an additional immunomagnetic separation (IMS) step was used following the manufacturer's instructions (Dynabeads<sup>®</sup> anti-*Salmonella*, Dynal A.S., Norway).

For the enrichment of *E. coli* O157, 20 ml of modified TSB (CM0985 containing VCC supplement SR0190) was added to 5 ml of sample, shaken at 150 rev min<sup>-1</sup> at 37 °C for 6 hours before streaking onto duplicate plates of Sorbitol MacConkey agar amended with cefixime-tellurite (CT-SMAC) in Trial A. In Trial B, IMS enrichment was also undertaken using Captivate<sup>™</sup> beads (Lab M, UK) with a slight modification of the manufacturer's instructions, i.e. using the enrichment procedure outlined above in order to maintain consistency with the enrichment of *E. coli* O157 in Trial A.

#### 2.6.1.3. Metabolic activity of *E. coli* O157

The degree of bioluminescence expressed by bacteria containing the *lux* gene directly indicates their metabolic activity, which represents a prerequisite for host infection (Unge et al., 1999). The metabolic activity of the *lux*-marked strain of *E. coli* O157 was measured by analysing 1 ml of the liquor in a SystemSURE 18172 luminometer (Hygiena Int., UK), and the bioluminescence values recorded as relative light units (RLU). For the biofilter samples, a sub-sample was mixed with sterile distilled water (1:5 (weight:volume)) and shaken for 5 minutes at 200 rev min<sup>-1</sup> before 1 ml of the supernatant was used for the analysis.

#### 2.6.1.4. 16S rRNA gene sequencing of presumptive *E. faecalis* isolates

A range of presumptive *Enterococcus* spp. colonies from Trial A were analyzed further using 16S rRNA gene sequencing. This was to confirm whether colonies bearing similar morphology to the inoculated strain of *E. faecalis* were indeed the introduced strain; thereby allowing us to determine the fate of the inoculated strain. Several additional isolates that were not presumptive *Enterococcus* spp., but which also grew on Slanetz and Bartley Medium (SBM; CM0377) agar were also sequenced. Frozen sub-samples (section 2.5) were defrosted, washed three times in MRD and grown on SBM. Twenty-four colonies of various morphologies from each time point were sub-cultured onto fresh SBM along with 10 colonies of pure culture. Isolated colonies were chosen for 16S rRNA gene-specific PCR using the

universal bacterial primers; forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Eurofins, Germany) (Furushita et al., 2008). Each 50 µl reaction mix consisted of 5 µl of PCR buffer and 0.1 U Taq DNA Polymerase, 0.2 mM mixed dNTP, 30 nM primer 27F, 30 nM primer 1492R, 36.5 µl H<sub>2</sub>O, and 1 bacterial colony. Other than the primers, all reagents were obtained from Roboklon GmbH (Germany). PCR reactions were run on the Tetrad 2, Peltier Thermal Cycler (Bio-Rad, UK) using the following conditions: initial denaturation step at 95 °C for 5 minutes followed by 34 cycles of 1 min denaturation at 95 °C, 1 min annealing at 50 °C and 2 min extension at 72 °C and a final extension step at 70 °C for 10 min. The ~1.5 kb products were visualised on a 1% agarose gel before being purified using the GeneMatrix DNA purification kit (Roboklon, GmbH) and sent for sequencing (in both directions) at Macrogen Inc. (South Korea). Sequence reads were assembled into contiguous sequences using Geneious Pro<sup>TM</sup> v5.4 (Drummond et al., 2011); the 1093 bp sequences were aligned using Clustal X v2.0.12 (Larkin et al., 2007). Poor quality sequences were removed from the alignment and a neighbour joining tree was constructed using 1,000 bootstraps and the Olsen correction in ARB v5.1 (Ludwig et al., 2004).

#### *2.6.2. Detection and quantification of PPV infectious virus and PCR measurement of viral load*

A 1:10 (w:v) suspension of each liquor and biofilter sample was prepared in virus transport medium and vortexed briefly, freeze-thawed for three cycles and centrifuged (3,000 rev min<sup>-1</sup>). Supernatants were stored at -80 °C until used. The MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics GmbH, Germany) was used to extract and purify the nucleic acid from bioreduction samples using a Roche MagNA Pure LC instrument (Roche Diagnostics GmbH) according to the manufacturer's instructions. The abundance of PPV in samples was determined using quantitative PCR. qPCR assays contained standard primers (forward primer, 5'-GGGGGAGGGCTTGTTAGAATCAC-3' and reverse primer 5'-ACCACACTCCCCATGCGTTAGC-3' amplifying a fragment of 155 bp in length) (Wilhelm et al., 2006), 1x Quantitect SYBR green Master Mix (Qiagen Ltd., UK) and 3 µl of template DNA or water for controls in a total reaction volume of 25 µl. A Roche LightCycler 480 (Roche Diagnostics GmbH) was used for all qPCR assays using the following conditions: initial denaturation step at 95 °C for 15 minutes followed by forty cycles of denaturation at 95 °C for 30 s; annealing at 60 °C for 30 s and extension at 72 °C for 30 s. A standard curve

was generated using a TOPO TA plasmid (Life Technologies, UK) containing a PCR product representing the entire PPV genome.

To establish the presence of infectious virus, 0.5 ml of each 1:10 (w:v) suspension was inoculated into a T25 flask containing a semi-confluent monolayer of primary pig kidney cells. Control flasks remained uninoculated. All flasks were monitored daily for 6 days for evidence of CPE. All flasks were then frozen and thawed three times and 1 ml inoculated into fresh flasks of semi-confluent primary pig kidney in order to establish if any CPE observed was being caused by initial toxicity of the bio-reduction sample rather than active virus. This passage was repeated twice more in order to allow any low levels of virus, which would otherwise go undetected, to multiply. If no CPE was present after these passages then samples were concluded to be negative for infectious virus.

To confirm the presence of PPV, flasks were freeze-thawed three times and 100 µl of cell lysate were added to a 24-well plate containing sterile glass cover slips and freshly trypsinised pig kidney cells. Cell cultures were left to incubate in 5% CO<sub>2</sub> at 30 °C for 48 h. Coverslips were harvested, fixed in acetone and then stained by indirect immunofluorescence (IIF) using a PPV specific monoclonal antibody and a mouse FITC labelled secondary antibody. Corresponding 1:10 (w:v) suspensions from those flasks confirmed positive by IIF were titrated to determine the amount of virus present in the original sample. These suspensions were titrated 10-fold and inoculated into 24-well plates containing a sterile glass cover slip and freshly trypsinised pig kidney cells (4 wells per dilution and 100 µl per well) and again left to incubate for 48 hours, stained and counterstained as described previously. Titres of original undiluted samples were calculated using the Spearman-Kärber method. Samples positive after passage in flasks but negative by titration of original sample were reported as positive, but with less infectious virus than could be quantified by virus isolation.

## *2.7. Bioaerosol analysis*

Bioaerosol samples were taken from the opening hatch of the vessels, above the centre of the biofilter, and 10 m upwind of the vessels, using selective agar within an Andersen Sampler as described in Gwyther et al. (2012). Industrial methylated spirit (80%) was used to disinfect the sampler between samples.

## 2.8. Data analysis

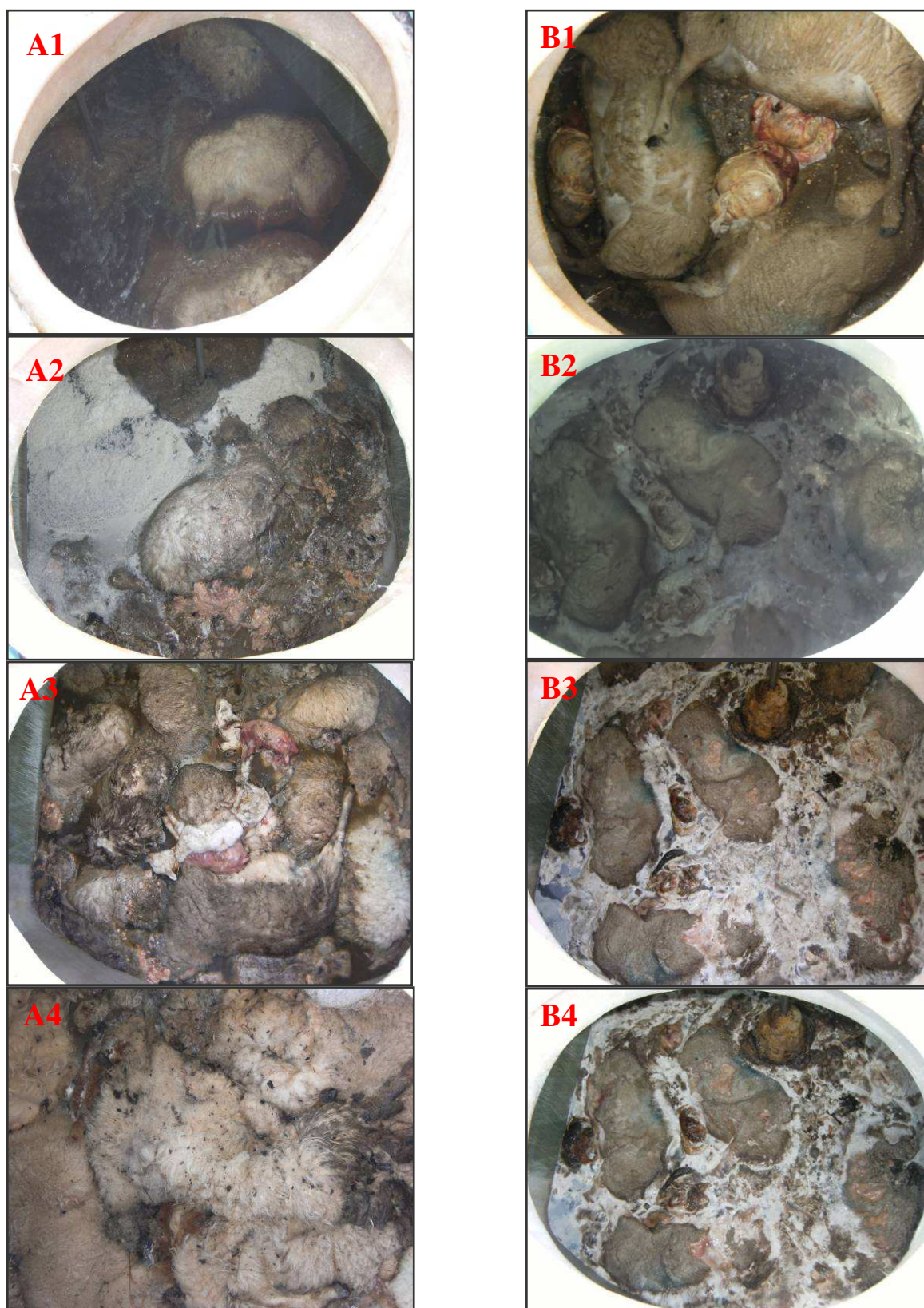
Colony counts were log transformed using the  $\log(x + 1)$  function. Samples that were analysed by enumeration only and were not detected were given an arbitrary value equal to half the detection limit of that method, whilst those not isolated by enumeration but were detected using enrichment were given an arbitrary value equal to the detection limit. Data were analysed using SPSS v15.0 and visualised using SigmaPlot v8.0 (SPSS Inc, USA). Paired Samples *t*-tests were used to analyse start and end counts whereas Independent Samples *t*-tests were used to analyse the relationships between treatments. Bioaerosol counts were corrected using the positive hole correction method (Macher, 1989).

## 3. Results

### 3.1. Trial management

No additional water was needed in either trial to ensure that carcasses were two-thirds covered. After the first month in Trial A, the majority of the carcasses had been degraded, allowing for further additions of carcass material as and when they died on farm (mostly full-sized sheep, and some young lambs). In contrast, the decomposition of carcasses in Trial B was retarded to the point where no further additions of carcasses were possible and whole carcasses were still identifiable by the end of the trial (Fig. 1)





**Figure 1:** Carcass breakdown in bioreduction vessel 1 under normal farm conditions (A) and simulated breakdown conditions (B). The different numbers represent different time points 1 = day 0, 2 = day 28, 3 = day 82 and 4 = day 127.

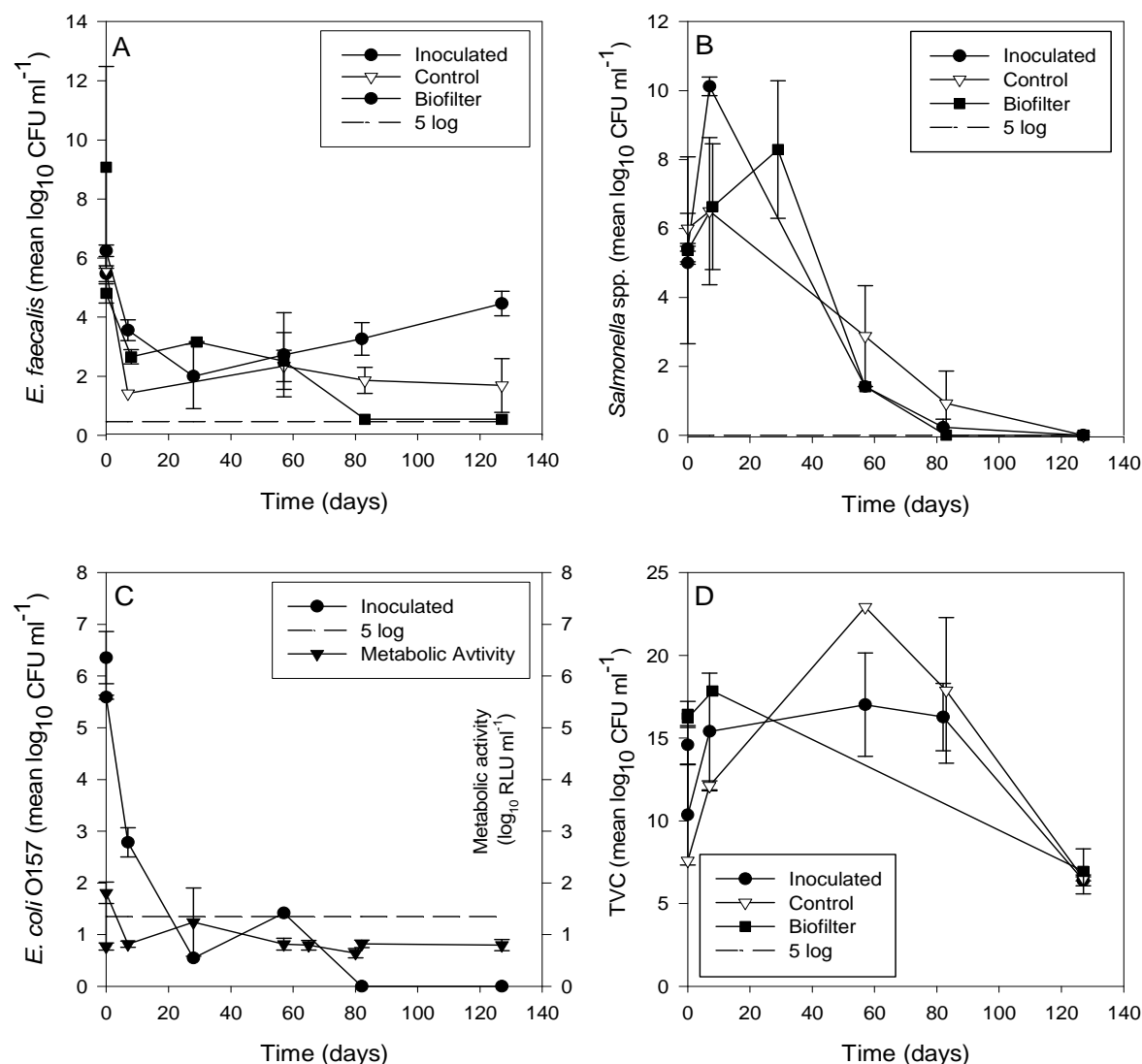
### 3.2. Microbial survival in liquor and biofilter

#### 3.2.1. Bacterial pathogens

##### 3.2.1.1. Trial A

Numbers of all bacterial pathogens declined in both the inoculated and the control vessels (Fig. 2), although responses varied between different micro-organisms. Culturing methods revealed an initial sharp decrease ( $P < 0.05$ ) for *E. faecalis* in inoculated vessels with a subsequent progressive and non-significant increase ( $P > 0.05$ ) until the end of the trial. However, the phylogeny of *Enterococcus* spp. isolated showed that whilst *E. faecalis* was detected by 16S rRNA analysis at the early stages of the trial, the number of isolates that were confirmed as positive declined to zero on day 7 (Fig. 3). Another spike in *E. faecalis* occurred on day 28 (43% of isolates confirmed as *E. faecalis*) and continued to decline again with only one colony confirmed as being *E. faecalis* on day 82 and none on day 127. All DNA sequences obtained in this study have been submitted to Genbank (accession numbers: JQ739617 – JQ739694). *Salmonella* spp. showed similar significant reductions in numbers in the inoculated vessels and controls ( $P < 0.01$  and  $P < 0.05$ , respectively), with numbers in the inoculated BVs decreasing by 5-log values. *E. coli* O157 was isolated from both control and inoculated vessels. Numbers in both treatments reduced to below detection by enrichment by day 82 (data not shown for controls). Further, *E. coli* O157 metabolic activity (as measured by RLU values) decreased to background levels from day 28 onwards in the inoculated vessels. TVC values in the control vessels increased as carcasses were added, but decreased significantly ( $P < 0.001$ ) towards the latter stages of the experiment (Fig. 2D). TVCs in the inoculated BVs showed a similar pattern of survival but although the decrease wasn't as pronounced by the end of the trial, it was still statistically significant; i.e. showed a >8-log reduction ( $P < 0.05$ ).

High background numbers of all microbes tested were found in the biofilter samples. Indeed, TVC and *E. faecalis* values were higher than in the inoculated liquor samples at time 0 (Fig. 2). The survival of these microbes showed similar patterns to the microbes in the liquor with a general reduction in numbers over time. Indeed, by day 82, *E. faecalis* numbers had reduced by approximately 8.5-logs (Fig. 2A) and there was also a 5-log reduction in numbers of both *Salmonella* spp. (Fig. 2B) and *E. coli* O157 (data not shown).

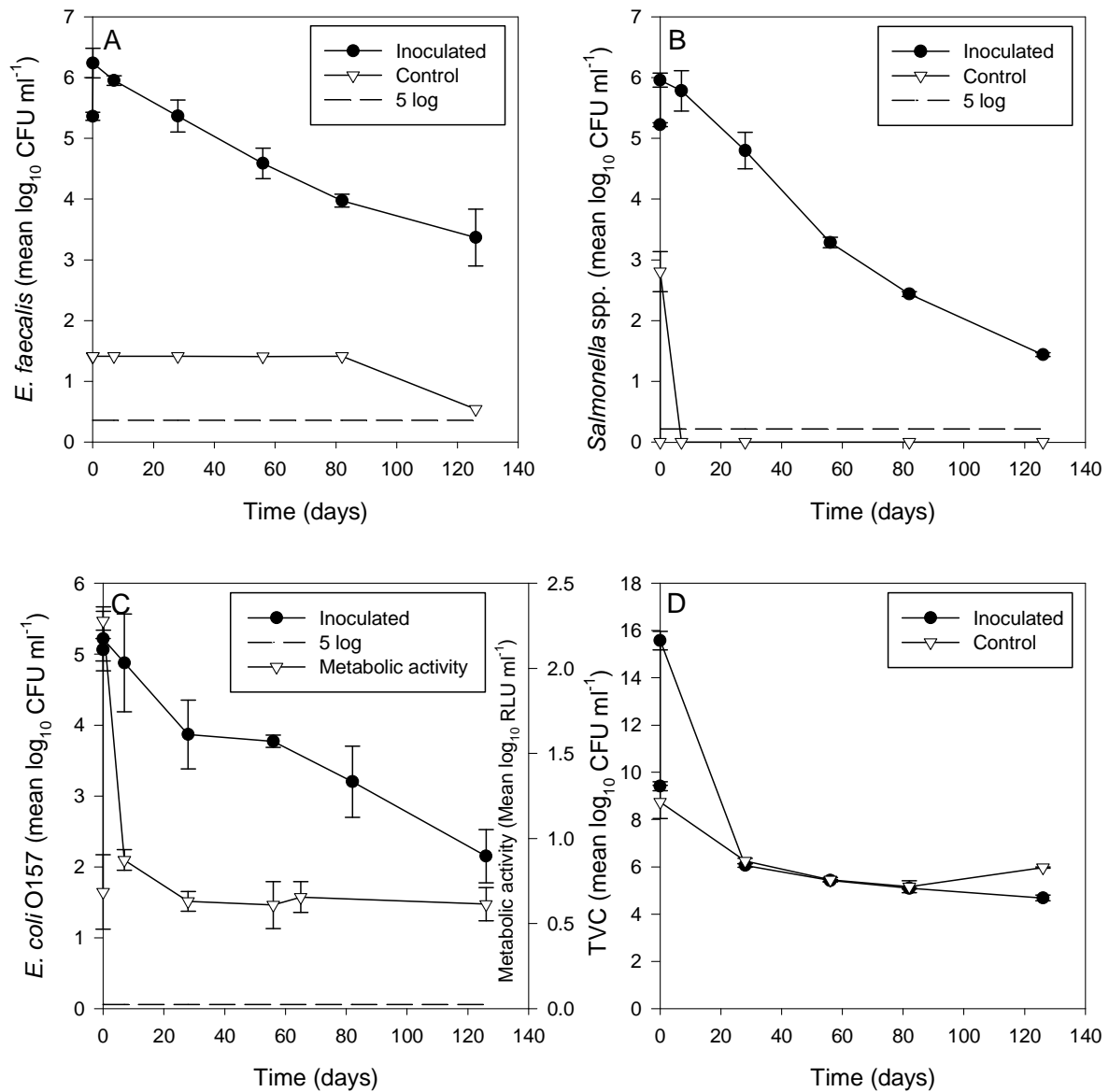


**Figure 2:** Numbers of *E. faecalis* (A), *Salmonella* spp. (B), *E. coli* O157 (C) and TVC (D) in inoculated and control vessels and the biofilter during Trial A. The dashed line represents a 5-log reduction from starting concentrations. Metabolic activity of *E. coli* was measured using bioluminescence and recorded as relative light units (RLU). Values represent means  $\pm$  standard error for the bio-reduction vessels and standard error of pseudo-reps for the biofilter ( $n = 1$ ).

### 3.2.1.2. Trial B

All bacterial pathogens were detected in the liquor throughout the 127 day period (Fig. 4) and none of the pathogens tested met the 5-log reduction target. However, all showed a significant decrease in numbers: *E. faecalis* a 2-log reduction ( $P < 0.05$ ), *E. coli* O157 a 2.9-log reduction ( $P < 0.05$ ) and *S. Senftenberg* a 3.8-log reduction ( $P < 0.01$ ). TVCs showed a





**Figure 4:** Numbers of *E. faecalis* (A), *Salmonella* spp. (B), *E. coli* O157 (C) and TVC (D) in the inoculated vessels and the biofilter during Trial B. The dashed line represents a 5-log reduction from starting concentrations. Metabolic activity of *E. coli* was measured using bioluminescence and recorded as relative light units (RLU). Values represent means  $\pm$  standard error for the bioreduction vessels and standard error of pseudo-reps for the biofilter ( $n = 1$ ).

### 3.3. Detection and quantification of PPV infectious virus and measurement of viral DNA

PPV viral DNA was detected in the bioreduction vessels by qPCR throughout both trials. However, infectious virus as measured by CPE and IIF showed over a 3- log reduction by day 7 for Trial A (Table 2). During Trial B, infectivity remained constant between 3.83 and 3.50 log<sub>10</sub> TCID<sub>50</sub> ( $P > 0.05$ ) thus a 3-log reduction didn't transpire (Table 2). Control vessels were negative for the presence of PPV by qPCR. No PPV DNA was detected in the biofilter in either of the trials.

**Table 2:** Detection and enumeration of porcine parvovirus from liquor samples in the inoculated bioreduction vessels during Trial A and Trial B. Values represent the mean  $\pm$  standard error ( $n = 3$ ). Blank cells = sample not taken.

Day	Trial A		Trial B	
	qPCR (log <sub>10</sub> copies ml <sup>-1</sup> )	CPE (log <sub>10</sub> TCID <sub>50</sub> )	qPCR (log <sub>10</sub> copies ml <sup>-1</sup> )	CPE (log <sub>10</sub> TCID <sub>50</sub> )
0		5.02 $\pm$ 0.0		4.00 $\pm$ 0.0
0.04	6.05 $\pm$ 0.41	4.17 $\pm$ 0.1	5.64 $\pm$ 0.33	3.50 $\pm$ 0.0
7	5.44 $\pm$ 0.17	1.00 $\pm$ 1.0	5.26 $\pm$ 0.06	3.83 $\pm$ 0.3
28	5.08 $\pm$ 0.37	1.00 $\pm$ 1.0	5.18 $\pm$ 0.05	3.83 $\pm$ 0.3
56	4.62 $\pm$ 0.64	0.00 $\pm$ 0.0	5.07 $\pm$ 0.12	3.50 $\pm$ 0.0
82	4.43 $\pm$ 0.62	0.00 $\pm$ 0.0	5.19 $\pm$ 0.06	3.50 $\pm$ 0.0
127			5.06 $\pm$ 0.05	3.83 $\pm$ 0.3

### 3.4. Bioaerosol analysis

#### 3.4.1. Trial A

Only low numbers of pathogens were isolated from bioaerosols released from the opening hatch of the inoculated vessels, with *E. faecalis* only detected an hour after inoculation (9.0 CFU m<sup>-3</sup>) and *Salmonella* spp. up until day 8 (2.2 CFU m<sup>-3</sup>). No *E. coli* O157 was detected at any time point. Within control vessels, no pathogens were detected as bioaerosols (Table 3). TVC numbers peaked at day 58 for inoculated vessels and day 8 for

control vessels, and soon returned to background levels. Only low numbers of *E. faecalis* and *Salmonella* spp. were detected from bioaerosols above the biofilter (3.3 CFU m<sup>-3</sup> on day 0.04 and 3.3 CFU m<sup>-3</sup> on day 8, respectively). TVC numbers from the biofilter bioaerosols were lower than those from the inoculated vessels and never reached numbers higher than those designated as ‘uncontaminated air’ by Breza-Boruta and Paluszak (2007), i.e. < 1,000 CFU m<sup>-3</sup> (Table 3).

#### 3.4.2. Trial B

Neither *E. faecalis* nor *E. coli* O157 were detected in bioaerosol samples during trial B and *S. Senftenberg* was only detected on day 0.04 (1.1 CFU m<sup>3</sup>) (Table 3). Unlike Trial A, TVC numbers peaked on the first sampling point and decreased to background levels by day 28 (background data not shown). There was a slight rise in TVCs on day 127 for the inoculated BVs, which were higher than the associated background numbers taken 10 m upwind but not significantly so ( $P > 0.05$ ; data not shown). Nevertheless, at no point in the trial did TVC rise above those categorised as ‘uncontaminated air’ (Table 3) (Breza-Boruta and Paluszak, 2007). Similarly for samples taken above the biofilter, TVC levels did not rise above 51.3 CFU m<sup>-3</sup> and so were well within the limits designated as ‘uncontaminated air’ (Breza-Boruta and Paluszak, 2007). Neither *E. faecalis*, *Salmonella* or *E. coli* O157 were isolated from aerosols generated by the biofilter (data not shown).

**Table 3:** Detection of bioaerosols from the bioreduction vessels (mean CFU m<sup>-3</sup> ± standard error), where BV = inoculated bioreduction vessels (*n* = 3) and CONBV = control bioreduction vessels (*n* = 2); - = none detected, blank = sample not taken. The contaminated air classification for Total Viable Counts is taken from Breza-Boruta and Paluszak (2007).

Day	<i>E. faecalis</i>			<i>Salmonella spp.</i>			<i>E. coli</i> O157			Total Viable Counts		
	Trial A		Trial B	Trial A		Trial B	Trial A		Trial B	Trial A		Trial B
	BV	CONBV	BV	BV	CONBV	BV	BV	CONBV	BV	BV	CONBV	BV
0.04	9.0 ± 7.4	-	-	1.1 ± 1.1	-	1.1 ± 1.1	-	-	-	4.4 ± 2.2	-	42.8 ± 3.0
8	-	-	-	2.2 ± 2.2	-	-	-	-	-	1469.4 ± 842.7	917.3 ± 511.3	11.1 ± 1.1
29	-	-	-			-	-	-	-			3.3 ± 3.3
58	-	-	-	-	-	-	-	-	-	2922.2 ± 2918.9	3.3 ± 0.0	-
85	-	-	-	-	-	-	-	-	-	1.1 ± 1.1	-	-
127	-	-	-	-	-	-	-	-	-	1.1 ± 1.1	3.3 ± 0.0	10.0 ± 1.9



<1000 CFU m<sup>-3</sup> – uncontaminated air



1000-3000 CFU m<sup>-3</sup> – moderately contaminated air



>3000 CFU m<sup>-3</sup> – highly contaminated air



## 4. Discussion

The results of this field-scale study support the conclusion of previous work that has shown active bioreduction to be effective at reducing the amount of animal waste for subsequent disposal (Williams et al., 2009). During the current Trial B, it was seen that having no active heating or aeration decreased the rate of decomposition of carcasses significantly (Fig. 1). Nevertheless, if bioreduction is approved for industry use, it is envisaged that farmers may periodically switch off the heating and aeration to save on running costs; vessels may also undergo no active aeration or heating during periods of breakdown or servicing. As a new method of storing livestock carcasses should not cause the proliferation of risks prior to disposal (Anon, 2009; EFSA, 2008), it was therefore important to determine the fate of pathogens in bioreduction vessels under both optimal/operational and sub-optimal (simulated breakdown) conditions/when switched off. Our results showed that numbers of all pathogens in the liquor reduced from their initial starting levels under both optimal and sub-optimal conditions; and in the case of *Salmonella* spp., *E. coli* O157 and PPV in Trial A, these reductions met the criteria for ratifying new disposal methods for EU ABPR (i.e. 5-log reduction for pathogenic bacteria and a 3-log reduction for viruses (Bohm, 2008)). Bioreduction however is an interim storage method *prior to* disposal via incineration or rendering (Williams et al., 2009). Provided that pathogens do not proliferate within, or propagate from, bioreduction vessels, then the risk of human infection from their use is therefore notionally low as any pathogens resident within the waste would be destroyed during subsequent incineration in any case.

Following the initial decrease, the subsequent increase of *E. faecalis* numbers in vessels during Trial A was hypothesised to be due to the additions of fresh carcasses into the vessels and the subsequent release of new *E. faecalis*, rather than the growth of the inoculated strains. Fresh additions of carcasses were added on day 16, and the molecular analysis demonstrated that the increase by day 28 was due to an increase in *Enterococcus* spp. in general whilst *E. faecalis* numbers actually decreased. Indeed, no *E. faecalis* isolates were isolated from the 16S rRNA analysis on day 127 of the trial, therefore indicating that a 5-log reduction in *E. faecalis* numbers did actually occur. In a lab trial simulating field conditions, Gwyther et al. (2012) showed that without the input of fresh carcass material, *E. faecalis* showed over a 4-log reduction after 3 months. Similarly, in Trial B of this study, again where no fresh carcasses were inputted, the decline of *E. faecalis* was gradual but continual. The 16S rRNA analysis performed showed the phylogeny of *Enterococcus* spp. to be split into eight groups,

the two major groups being the *E. faecalis* and the *E. faecium* groups; similar to the description by Franz et al. (2003). At the start of Trial A, the majority of colonies isolated belonged to the *E. faecalis* group, whereas after day 7 the majority were grouped with *E. faecium* (Fig. 3). It is possible that the sheer numbers of *E. faecalis* inoculated into the bioreduction vessels hid these populations in earlier samples, but it is more likely that the *E. faecium* group microbes had not yet been released from the carcasses inputted at the start of the experiment (day 0).

The metabolic activity of *E. coli* O157 within waste matrices has been shown to vary (Williams et al., 2008a, 2008b). Bioluminescence measurements showed that there was a decrease in the metabolic activity of *E. coli* O157 during bioreduction. Given the association between metabolic activity and infectivity (Unge, 1999), this adds further evidence that bioreduction reduces any threat posed by zoonotic pathogens.

Very low numbers of pathogens were isolated in bioaerosols. Indeed, when the tanks were fully aerated and therefore most likely to lead to aerosolisation of pathogens, *Salmonella* spp. and *E. faecalis* were only isolated from the loading hatches, and immediately after inoculation. Using the classification system proposed by Breza-Botura & Paluszak (2007), TVCs from air within the inoculated vessels in Trial A would only be classified as 'moderately contaminated' on two separate occasions (day 8 and 58). Further, at no point in the control vessels in Trial A, or at all in Trial B, were TVC higher than in 'uncontaminated air' (Table 3). Samples were taken continuously over a 30 minute period (at a rate of 10 l min<sup>-1</sup>), whereas during real on-farm use it is unlikely that the bioreduction vessels would remain open for more than 2-3 minutes at a time, e.g. during carcass loading. Consequently, we envisage that the numbers of bacteria isolated represent an overestimation of those that would be encountered during manual operation of a bioreduction vessel.

In contrast to the previous field trial on bioreduction (Williams et al., 2009), there were no recorded incidences of malodour during the current trial. This is due to the inclusion of a compost: woodchip biofilter through which all exhaust pipes were vented, causing the adherence of odorous molecules to the organic fraction (Sheridan et al., 2003). Although it may be envisaged that the biofilter represented a potentially high risk point due to the continued release of bioaerosols from all five vessels, all biofilter samples showed a reduction in pathogen numbers over time. Indeed, numbers of background microbes resident within the biofilter material notably reduced during the trial, probably due to active aeration of the pile. Under on-farm conditions, it is envisaged that only one bioreduction vessel would

be employed and therefore the risk of bioaerosol contamination of the biofilter would be reduced. Although Verreault et al. (2008) states that viruses can be aerosolised, particularly during forced aeration, no PPV DNA was detected by qPCR in the biofilter during Trial A. This suggests that PPV was either inactivated before it was carried through to the biofilter, deposited in the pipes leading to the biofilter, inactivated whilst at the biofilter or that the rate of aeration was insufficient for aerosolisation. The latter explanation is the most likely as during the early stages of the trial, PPV within the liquor remained infective.

Johansson et al. (2005) hypothesised that the persistence of PPV in soil treated with anaerobic digestion sludge was due to the virus adhering to soil particles; other studies have also shown that viruses resist degradation when adhered to organic matter (Rao et al., 1984). This may explain why infective PPV was isolated from liquor samples in Trial B throughout the trial period, although PPV was inactivated by day 56 in Trial A. Virus inactivation may be caused by a variety of processes, including aeration and microbial inactivation (Lund et al., 1983; Knowlton et al., 1987). Ward and Ashley (1977) also reported a virucidal effect of free ammonia in wastewater sludge at concentrations higher than 0.4 M. Total ammonia is converted to free ammonia at pH >8 (Pesaro et al., 1995), and as the average pH of sheep bioreduction liquor is greater than 8.5 (Williams et al., 2009), it is possible that free ammonia in the liquor will be acting as a virucidal agent. The inactivation of PPV during Trial A could therefore be due to the combined effects of aeration reducing the adherence of particles to protective organic matter, high numbers of antagonistic microbes, and virucidal concentrations of ammonia. It is also possible that PPV adhered to the walls of the vessels as the volume of waste reduced; thus potentially rendering the PPV difficult to isolate and enumerate. Evidence of this includes the PPV-positive background samples taken prior to the addition of carcasses and PPV inoculum in Trial B, despite the disinfection of the bioreduction vessels using hydrogen peroxide. However, PPV has been shown to be resistant to disinfection by hydrogen peroxide, amongst other disinfectants (Eterpi et al., 2009), particularly PPV that has dried on surfaces (Terpstra et al., 2007). If disinfection of bioreduction vessels post-emptying was deemed necessary as part of operational procedure, it is recommended that the vessels are thoroughly soaked with water beforehand to re-suspend any virus particles present (Terpstra et al., 2007).

These field trials provide comprehensive evidence that bioreduction is a contained system that does not lead to the proliferation of common zoonotic pathogens, under contrasting management regimes. The encouraging findings of this trial alongside a body of

other evidence (Adkin et al., 2010; Gwyther et al., 2012; Williams et al., 2009) should be considered positively by policy-makers when deciding whether to ratify bio-reduction for industry use.

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**The efficacy of various commercial accelerants at enhancing the bioreduction of carcass constituents**

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## ABSTRACT

Commercial accelerants are routinely sold as additives for biological waste disposal methods such as composting and wastewater treatment. The aim of this study was to determine whether such accelerants or natural alternatives improve the efficacy of bioreduction. Mini bioreduction vessels containing pork and water were incubated at mesophilic temperatures and aerated to represent bioreduction processes. Seven different accelerants were used: three from the Alken and Murray 'Clearflo' range; 4003, 4006 and 7003, Filterboost (JBL), Gel 60<sup>®</sup> (Biopolym), liquor from a previous bioreduction, and soil. Two dose rates of accelerant were tested: 0.25 g accelerant to 1 g pork (low dose) and 0.5 g accelerant to 1 g pork (high dose). Samples were analysed for mass loss, moisture content, protein and amino acid content and total microbial counts over a period of thirteen days. Accelerants were deemed to be successful if the overall mean of the mass loss and moisture content were significantly lower than the control and if protein and amino acids content were significantly higher (indicating breakdown of material). Although Gel 60<sup>®</sup> met some of the criteria; none of the accelerants yielded any notable increase in the rate of material breakdown. Further, the use of Gel 60<sup>®</sup> at the more effective high dose rate would incur a 500-fold increase in product use and cost per kg of carcass than the currently recommended dose. Our trial suggests that accelerant products are therefore unnecessary in a bioreduction system.

*Keywords:* additives; agriculture; carcass disposal; fallen livestock; organic waste.

## 1. Introduction

Bioreduction has the potential to become a novel method of storing animal carcasses on farm prior to disposal (Gwyther et al., 2011). During bioreduction, decomposition is driven by the activity of internal enzymes and microbial communities under mesophilic and aerobic conditions reducing the final volume of waste to be produced (Williams et al., 2009). However, in order to facilitate the initial stages of microbial degradation, a catalyst product Gel-60<sup>®</sup> (Biopolym, Spain), based on sodium alginate derived from brown seaweed (*Ascophyllum nodosum*), has been used during both anaerobic (Gutiérrez et al., 2003) and aerobic bioreduction experiments to date (Gwyther et al., 2012a, 2012b; Williams et al., 2009). It is claimed that the hydrolysis of the fibre, proteins, polymeric polysaccharides and oligosaccharides of Gel-60<sup>®</sup> provide ready substrates for the indigenous microbial community, encouraging microbial growth and bioreduction of the animal carcass (Gutiérrez et al., 2003); thus acting as an ‘accelerant’.

The use of accelerants in biotechnology is not a new concept with many products on the market claiming to catalyse the degradation of organic material. Investigations into the use of accelerants in composting processes, including the use of plant preparations (Carpenter-Boggs et al., 2000) and microbes (Chang and Yang, 2009; Gaind et al., 2005; Nair and Okamitsu, 2010; Tsai et al., 2007) suggest that additives may increase the rate of compost maturation. However, the use of mature compost as a starting inoculum is a common practice (Sundberg and Jönsson, 2005). Similarly for anaerobic digestion the use of inoculums from existing facilities, or the use of fresh manure, is often used to start new reactors (Gerardi, 2003).

The addition of an accelerant with the loading of each carcass or batch of carcasses presents an extra cost in the bioreduction process. For bioreduction to be seen favourably by the farming community, it is important that only essential procedures are included to make the process more user-friendly and cost-effective. The aim of this study was therefore to determine the effect of different accelerants on carcasses decomposition rates during bioreduction. The following products were tested in laboratory-scale bioreduction vessels: Alken Clearflo<sup>®</sup> 4003, 4006 and 7003 (Alken-Murray, 2011), products based on *Bacillus* strains in varying cocktails with other bacteria and/or enzymes used to clear food oil and grease from food processing wastewater; Filterboost<sup>®</sup> (JBL, 2011), a bacterial culture on a granulate material used to remove organic matter from the filters of fish tanks; Gel-60<sup>®</sup> (Biopolym, 2011); liquor from previously bioreduced porcine carcasses; and soil.

## 2. Methods

### 2.1 *Experimental design and sampling*

Mini bioreduction vessels (MBVs) were created using 50 ml centrifuge tubes. Pork (6 g) was added to each tube together with one of the accelerants either at a ratio of 0.25 g accelerant to 1 g pork in the low dose (low) treatment ( $n = 3$ ) or 0.5 g of accelerant to 1 g pork in the high dose (high) treatment ( $n = 3$ ). JBL Filterboost was bought from commercial suppliers whereas the Alken Murray products and Gel 60<sup>®</sup> were donated by their respective companies. The mature bioreduction liquor was obtained from bioreduction trials at Bangor University's Henfaes Research Station and soil (Brown Earth) from a nearby field. Control MBVs contained no additives ( $n = 3$ ). A total volume of 50 ml was made up using distilled water. The MBVs were incubated in the dark at 37 °C and aerated through shaking (200 rev min<sup>-1</sup>) to represent the conditions within the bioreduction vessels. Samples were taken on days 0, 1, 3, 6, 8, 10 and 13. A sample (3.1 ml) was removed from each MBV at every time point and MBVs were subsequently made up to 50 ml to prevent desiccation of the pork material.

### 2.2 *Chemical and biological analyses*

All bacteriological media were obtained from Oxoid (Oxoid Ltd., Basingstoke, UK). The MBVs were weighed on day 0 and before any liquor was removed for further analyses on subsequent sampling dates. Total viable counts (TVC) were measured by serially diluting 0.1 ml of liquor with Maximum Recovery Diluent (MRD; CM0733) and plating onto R2A agar (CM0906). Plates were incubated at 20 °C for 48 hours. The physical process of decomposition was measured by determining the moisture content and organic matter content of the dry weight portion according to standard techniques; samples were dried at 105 °C for 24 hours and weighed before ashing at 450 °C for a further 24 hours. The Bradford assay was used to determine the total protein content of the liquor (Bradford, 1976) using a 96-well plate spectrophotometer (BioTek<sup>®</sup>, Winooski, USA) and amino acids were determined using a fluorometric assay (Jones et al., 2002) with a Varian Cary Eclipse Spectrophotometer (Varian Inc., Palo Alto, USA). Samples were diluted with distilled water where necessary. A repeat trial was run for both the control and Gel 60<sup>®</sup> treatments analysing just mass loss and volume of liquor lost with samples taken on days 0, 1, 2, 5, 9 and 13. The volume of liquor

lost in this case was analysed by recording the volume of distilled water added to make up the 50 ml volume at the end of each sampling time.

### *2.3 Success criteria*

An accelerant was deemed to enhance the rate of decomposition if:

- 1) The mass of the mini bioreducers (MBVs) was significantly reduced to below the mass of the control MBVs,
- 2) Moisture content was significantly lower than the control MBVs, and
- 3) Both protein and amino acid concentration was significantly higher in treatments containing accelerants than control.

### *2.4 Data analysis*

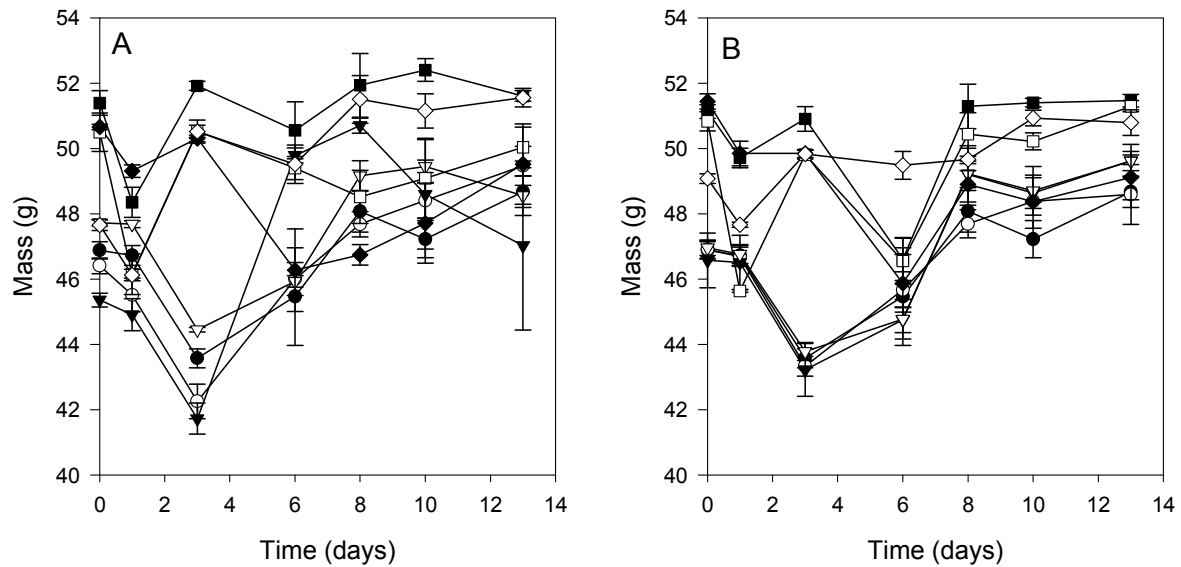
Data were analysed using SPSS v15.0 and displayed using SigmaPlot v8.0 (SPSS Inc, Chicago). Differences in each of the variables between the start and end points were analysed using paired samples *t*-tests, whereas data at each time point were compared using one-way ANOVA. Means of each treatment were compared to the control samples using independent samples *t*-tests.

## **3. Results**

Treatments 4006 (high and low), 7003 (high and low) and liquor (high and low) showed a decrease in mass until day 3 and a subsequent increase until day 13, consistent with the control treatment ( $P > 0.05$ ) (Fig. 1). JBL (high and low) had a higher average mean mass than control ( $P < 0.01$ ), but this may be explained by the mass of the granulate material that the bacterial inoculum is bound to. Similarly for soil, both high and low dose treatments were heavier than the control on days 0-3 ( $P < 0.01$ ) although by day 6 there was no significant difference ( $P > 0.05$ ). Treatments with both 4003 and Gel 60<sup>®</sup> had a higher overall mean than the control (all  $P < 0.001$ ) with an earlier peak in mass.

For the second mass loss trial (Fig. 2), control MBVs showed a significant decrease of 0.96 g ( $P < 0.05$ ) by the end of the trial, whilst neither Gel 60<sup>®</sup> high and low revealed any significant difference ( $P > 0.05$ ). Unlike the first trial, neither of the Gel 60<sup>®</sup> treatments

showed a significant difference in mass compared to the control with the exception of time 0 where Gel 60<sup>®</sup> high was significantly higher ( $P < 0.001$ ) and Gel 60<sup>®</sup> low was significantly lower ( $P = 0.001$ ). A comparison of mean mass of the two trials showed that the control values were significantly higher in trial 2 than trial 1 ( $P < 0.001$ ), Gel 60<sup>®</sup> high was significantly lower in trial 2 ( $P = 0.001$ ) and there was no significant difference between the two trials for Gel 60<sup>®</sup> low ( $P > 0.05$ ).

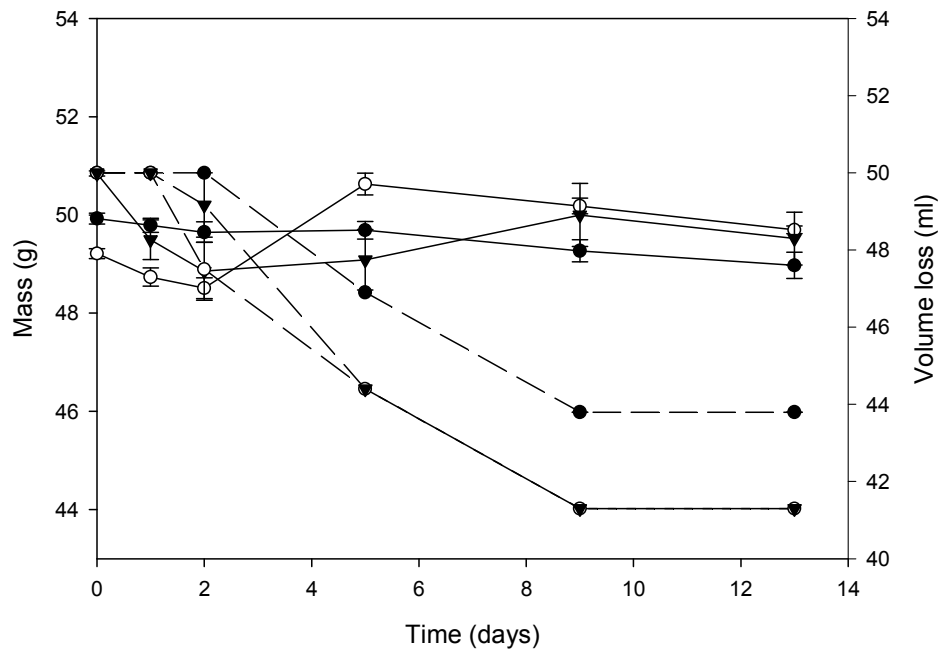


**Figure 1:** Mean mass at the high (A) and low (B) dose rates for each of the treatments: ● Control; ○ 4006; ▼ 7003; ▽ liquor; ■ JBL; □ 4003; ◆ soil and ◇ Gel 60<sup>®</sup>. Values represent means ( $n = 3$ )  $\pm$  the standard error of the means.

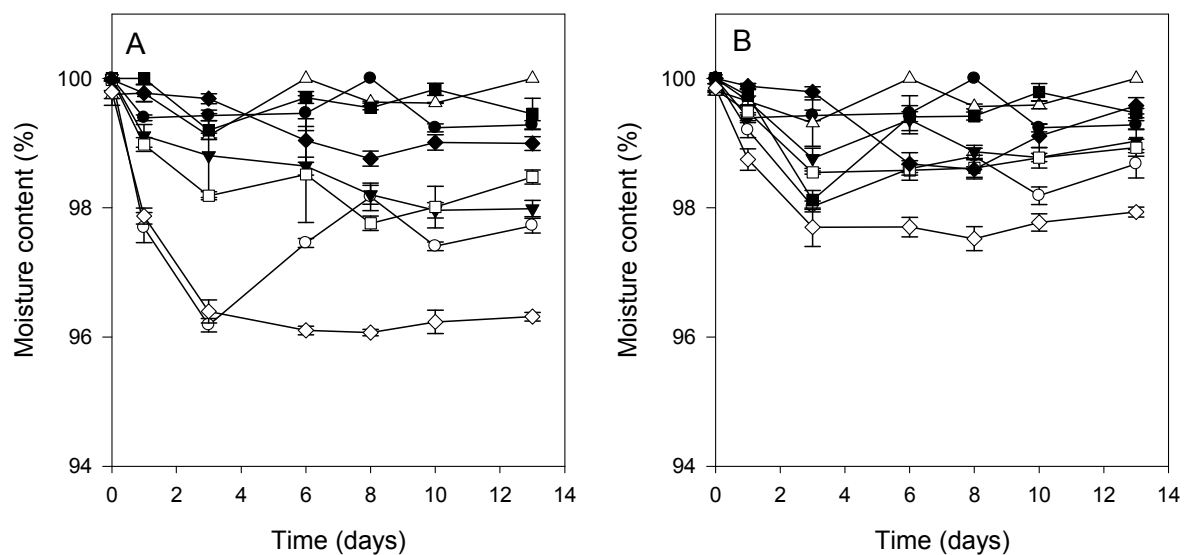
During the second mass-loss trial, a total of 6.2 ml of distilled water was added to the control MBVs and 8.7 ml of water added to each MBV containing Gel-60<sup>®</sup> over the course of the trial to maintain water levels at 50 ml. Therefore, treatments containing Gel-60<sup>®</sup> lost 17.4 % of liquid via evaporation throughout the 13 day trial whilst the controls lost only 12.4 %. Statistics were not able to be performed on this data as the standard deviations were 0.

Moisture content in the control treatments fluctuated throughout the trial, with a peak in moisture content on day 8 (Fig. 3). All treatments, with the exceptions of liquor (high and low), soil (high and low) and JBL high, showed lower overall mean moisture contents than the control MBVs ( $P < 0.05$ ). JBL low was only significantly lower than the control on day 3 ( $P < 0.05$ ) and soil (high and low) was significantly lower on day 8 ( $P < 0.01$ ). Treatments

containing liquor were not statistically different from control until day 13 when the control was statistically lower ( $P < 0.05$ ).



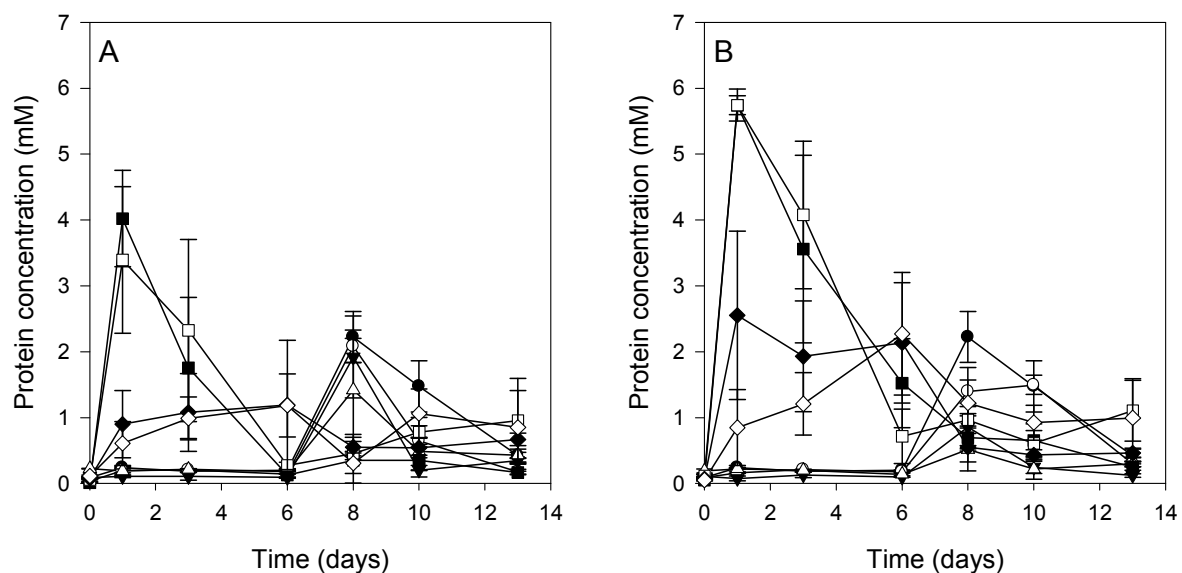
**Figure 2:** Results of the mass (continuous line) and volume (dashed line) loss in the second trial using the following treatments: ● control, ○ Gel 60<sup>®</sup> low dose and ▼ Gel 60<sup>®</sup> high dose. Values represent means ( $n = 3$ )  $\pm$  the standard error of the means.



**Figure 3:** Mean moisture content over time at the high (A) and low (B) dose rates for each of the treatments: ● Control; ○ 4006; ▼ 7003; ▽ liquor; ■ JBL; □ 4003; ◆ soil and ◇ Gel 60<sup>®</sup>. Values represent means ( $n = 3$ )  $\pm$  the standard error of the means.

Protein content in the control peaked on days 8 ( $P < 0.001$ ) and 10 ( $P < 0.05$ ) and returned to levels seen at the start of the trial by day 13 ( $P > 0.05$ ). Treatments 4003 (high and low), JBL (high and low), soil (high and low) and Gel 60<sup>®</sup> (high and low) all showed peaks in protein levels before the control (Fig. 4). 4003 (high and low) showed a sharp peak on day 1, whereas protein content in both soil treatments was lower but more sustained. Gel 60<sup>®</sup> showed a gradual rise rather than a sharp peak and reached maximum values on day 6, just 2 days before the peak in the control. The remaining treatments showed similar patterns to the control. 4003 low and JBL low had the highest protein concentrations (both 5.74 mM) and were the only treatments to have a significantly higher mean than the control ( $P < 0.05$ ).

The concentration of amino acids in the control MBVs showed a gradual increase until they peaked on day 10 with a mean maximum value of 59.6 mM ( $\pm 25.4$  mM). A similar pattern was observed for the rest of the treatments (Fig. 5) although the peak in the amino acid concentration varied from treatment to treatment. For example, JBL (high and low) and liquor (high and low) peaked on day 3. Nevertheless, only 4006 (high and low) and Gel 60<sup>®</sup> (high and low) had significantly higher means ( $P < 0.001$ ,  $P = 0.001$ ,  $P < 0.05$  and  $P = 0.01$ , respectively).

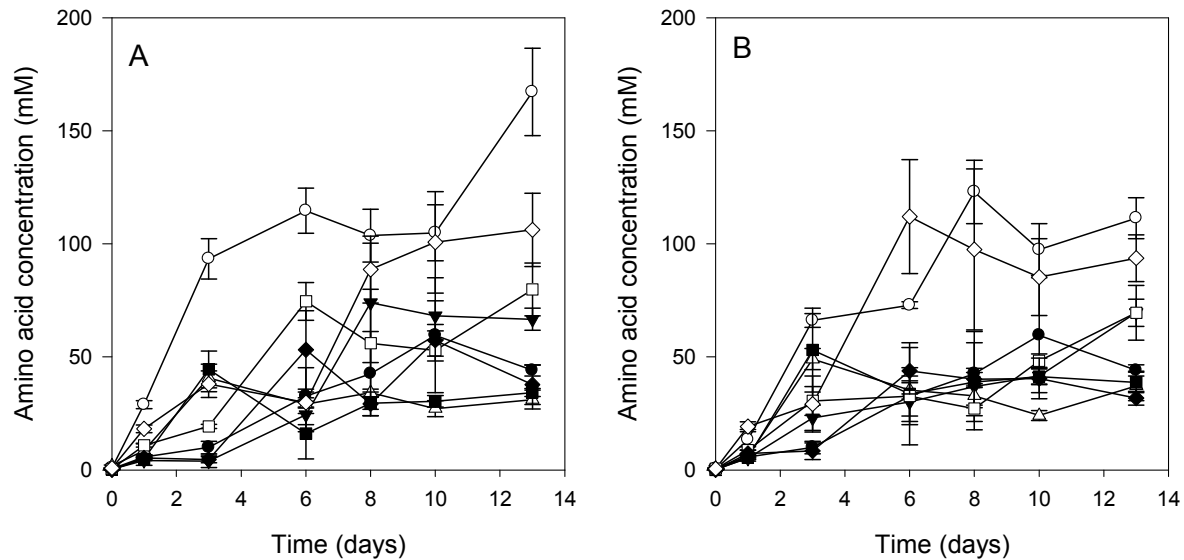


**Figure 4:** Mean protein concentration over time at the high (A) and low (B) dose rates for each of the treatments: ● Control; ○ 4006; ▼ 7003; ▽ liquor; ■ JBL; □ 4003; ◆ soil and ◇ Gel 60<sup>®</sup>. Values represent means ( $n = 3$ )  $\pm$  the standard error of the means.

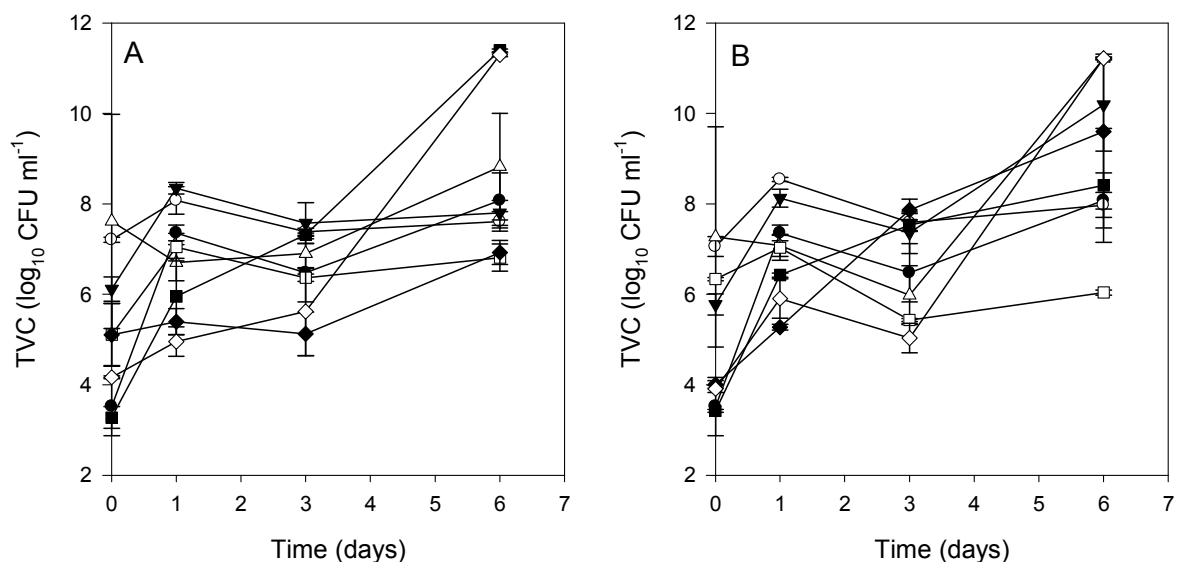
TVC data was available for days 0 to 6 only (Fig. 6). By the end of this 6 day period only JBL high and Gel 60<sup>®</sup> high and low showed a significant increase in TVC numbers (all



$P < 0.001$ ). Indeed, 4003 low showed a significant decrease in bacterial counts ( $P < 0.01$ ). Only 4006 low had a significantly higher mean than the control ( $P < 0.05$ ), although no treatments were statistically different by the end of the 6 days ( $P > 0.05$ ).



**Figure 5:** The mean concentration of amino acids over time at the high (A) and low (B) dose rates for each of the treatments: ● Control; ○ 4006; ▼ 7003; ▽ liquor; ■ JBL; □ 4003; ◆ soil and ◇ Gel 60®. Values represent means ( $n = 3$ )  $\pm$  the standard error of the means.



**Figure 6:** The numbers of total viable bacterial counts over time at the high (A) and low (B) dose rates for each of the accelerants: ● Control; ○ 4006; ▼ 7003; ▽ liquor; ■ JBL; □ 4003; ◆ soil and ◇ Gel 60®. Values represent means ( $n = 3$ )  $\pm$  the standard error of the means.

#### 4. Discussion

Mass loss has been used as a direct measure for decomposition processes in previous cadaver studies (Tibbett et al., 2004). During bioreduction, water is lost via evaporation resulting in a reduction in volume and therefore mass loss over time. With the removal of some of the liquor for analysis which may include some of the products of decomposition, it was hypothesised that this would further reduce mass loss despite the MBVs being replenished with fresh water after sample analysis was complete. In spite of an initial fall in mass and contrary to what we were expecting, the majority of treatments showed an overall increase by the end of the trial. Nevertheless this was only significant for 4006 high, 7003 low, liquor low and Gel 60 high<sup>®</sup> (all  $P < 0.05$ ). Experimental error may account for a change in recorded mass of up to 5% and all the aforementioned accelerants showed a greater than 5% change by the end of the trial. However, on day 3 where the control showed a big decline in mass (-7.6%), 4006 (high and low), 7003 (high and low) and liquor (high and low) all showed a decrease in mass of greater than 5%. Whilst the trend up to this point showed what was expected, the replicate trial for control and Gel 60<sup>®</sup> (high and low) failed to show such a considerable decrease. One possible explanation for the overall increase in mass is that as solid material dissolved in the liquor the volume of liquor decreased. Thus, as water was added to bring the volume up to 50 ml, the mass of the MBVs increased; although this doesn't explain the discrepancy in results between the two mass loss trials.

The average mass of the control MBVs in trial 2 was significantly higher than for trial 1 ( $P < 0.001$ ). This is due to the higher initial mass in trial 2 control MBVs and because the decline in mass loss was less severe than in trial 1. The difference in initial mass loss (6% higher in trial 2) may be explained by the differences in the laboratory set-up of the different trials. Differences in the surface area of the pork portions may have resulted in a difference in the volume of water added to the MBVs. The sharp decline in mass loss was recorded on day 3 for trial 1 whereas samples were taken on day 2 for trial 2 due to logistical problems. Therefore, the sharp decrease in mass may have been missed. Whatever the explanation for the differences in mass loss, this study does highlight the necessity for accurate replication of experiments.

An increase in organic matter content of the dry weight portion (data not shown) and a subsequent decrease in moisture content of the liquor over time were hypothesised as particulate material from the degradation of the pork enters solution and water is lost via evaporation. The control did indeed follow this pattern and showed a significant reduction in

moisture content by the end of the trial ( $P < 0.05$ ) as did all other treatments with the exception of 4006 high, liquor high & low, JBL high and soil low. Nevertheless, it should be noted that the moisture content does not reflect the total reduction in moisture content within the MBVs as water was replenished in each MBV after the sample volume was removed. It was assumed that the rate of evaporation from each vessel would be identical so would not produce changes in perceived patterns although water lost via evaporation was not directly measured. However, using our success criteria the largest difference from the mean values of the control is Gel-60<sup>®</sup> high with an average difference of 3.48% ( $P < 0.05$ ), followed by 4006 high (2.28%,  $P < 0.01$ ), 7003 high (2.01%,  $P < 0.01$ ) and Gel 60<sup>®</sup> low (1.92%,  $P < 0.01$ ).

Although this study was focused on mass loss associated with evaporation, it is likely that reductions were due in part to the loss of carbon from the MBVs through bacterial respiration of organic material as CO<sub>2</sub>. Future experiments could address this omission by simply comparing the total- and volatile solids contents of the entire MBV at the start of the trial to those at the end; or for a more advanced approach, by measuring microbial respiration throughout the trial. Various methods can be used to analyse microbial respiration in the laboratory and include the use of dedicated respirometers, or acid traps to capture the released CO<sub>2</sub>. Suitable methods commonly used soil science can be found in Alef and Nannipieri (1995).

The Bradford assay was chosen for the determination of proteins in solution despite the potential for interference by humic substances and phenolic compounds (Roberts and Jones, 2008) and the inability of this assay to analyse proteins  $< 3,000$  Da, as it is a simple, quick and inexpensive method (Moore et al., 2010). Detection of protein content in the liquid phase should reduce the measurement of proteins from the microbial biomass (Roberts and Jones, 2008) indicating how much protein has been released via microbial, enzymatic or physical degradation of the pork substrate. The early peak in protein concentration from 4003 (high and low), JBL (high and low), soil (high and low) and Gel 60<sup>®</sup> (high and low) indicates that the decomposition process was proceeding at a faster rate than in the control and other treatments (Fig. 2). Nevertheless by day 13 there was no longer a statistical difference in the concentration of protein between the control and any of the treatments ( $P > 0.05$ ).

Amino acid concentrations were not expected to be elevated until after the peak in protein concentration as the microbial consortia and enzymes break down the smaller peptides into their principal components. The exceptions to this were hypothesised to be soil and liquor as they have already undergone decomposition processes. Nevertheless, initial

values suggest that the amino acids in these substrates have already been utilised by their respective microbial populations as they are not statistically different from the control on day 0, with the exception of liquor high ( $P < 0.05$ ). 4006 high and low showed the highest concentrations of amino acids with Gel 60<sup>®</sup> high and low coming in third and fourth.

Given the assumption that accelerants such as Gel 60<sup>®</sup> are supposed to catalyse the early stages of decomposition by providing a ready source of nutrients for the microbial community (Gutiérrez et al., 2003), it is surprising that there were no significant differences in overall TVC numbers between the control and the rest of the treatments (with the exception of 4006 low), particularly for those treatments that contained an active bacterial consortia. Whilst total viable counts cannot be used as a direct measurement of bacterial activity we would still expect the culturable fraction of TVCs to be higher in the treatments than the controls if degradation of the pork substrate was faster and nutrients more readily available for bacterial growth. Without measuring activity directly such as using the dehydrogenase enzyme assay or TVC counts over a longer period of time, we cannot say for certain whether the accelerants are having any appreciable effect.

Of all the accelerants tested, Gel 60<sup>®</sup> met the most requirements of our success criteria, although in the case of mass it showed the highest increase rather than decrease. Nevertheless it must be pointed out that by day 13 neither mass or protein concentration were significantly different to the control indicating that the degradation process was beginning to equalise despite the bulk of the pork substrate still remaining. With regards to dose rate, there was no significant difference between the values of mass, protein concentration and amino acid concentration between the two treatments although moisture content in Gel 60<sup>®</sup> low was statistically higher ( $P=0.01$ ) suggesting that the high dose rate was more efficient at breaking down the pork. The recommended dose rate by the manufacturer is 0.001 g Gel 60<sup>®</sup> to 1 g carcass. This dose rate is substantially lower than the dose rates used in this trial and therefore it is highly unlikely that using such a low dose of the product would produce any appreciable benefits in the rate of bio-reduction. Using Gel 60<sup>®</sup> at this high dose rate would incur a 500 fold increase in the amount of accelerant to be used per carcass and therefore a 500 fold increase in cost.

None of the accelerants used in this experiment were aimed specifically at enhancing bio-reduction, with the possible exception of Gel 60<sup>®</sup>, although they were designed to reduce the organic matter content of food waste in water (Alken Murray, 2011; JBL, 2011). Without tailoring the particular bacterial consortia or enzymes to specific livestock species it is

unlikely that any accelerant will have any effect during bioreduction. Nevertheless, the liquor from a previous swine carcass bioreduction also failed to meet any of the success criteria. This was unexpected as bioreduction liquor has been recommended to be used as a starting inoculum for further bioreductions (Lobera et al., 2007) and potentially contains all the enzymes and microbes needed to facilitate bioreduction. However, since the liquor used was from the end of a bioreduction cycle, it is possible that the decomposition process had slowed or stopped, highlighting the need for further research in this area.

## **5. Conclusions**

Commercial and other accelerants failed to enhance the rate of bioreduction, even at application rates significantly higher than those recommended. Although the findings of this trial indicate that there is no direct benefit in using existing catalyst products for bioreduction, new and cost-effective bacterial and enzymatic products should be developed to further enhance the efficiency of bioreduction.

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**A comparison of the carbon footprints of conventional and novel systems for the storage and disposal of animal carcasses**

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## **ABSTRACT**

Greenhouse gasses (GHG) released during the decomposition of animal carcasses are generally ignored when formulating whole-farm carbon footprints. The carbon footprints of conventional methods of carcass storage/disposal were analysed following standard Life Cycle Assessment techniques and compared to bioreduction, a novel method of storing fallen stock prior to ultimate disposal. Over a one year period, bioreduction had the highest footprint (10,139 kg CO<sub>2</sub>e) followed by burial (4,254 kg CO<sub>2</sub>e), freezing followed by collection (3,758 kg CO<sub>2</sub>e), conventional collection using a central collection service (194 kg CO<sub>2</sub>e) and finally conventional collection using ‘Dolav’ storage boxes to prolong the time between collections (155 kg CO<sub>2</sub>e). Although bioreduction may look unfavourable from a GHG perspective, within a typical whole-farm footprint it would still account for <2% of the total farm’s GHG emissions. Further, the benefits of improved biosecurity associated with bioreduction may outweigh the high carbon footprint.

## **Keywords**

Agriculture; environmental impact; livestock mortalities; waste management.

## 1. Introduction

Globally, the livestock industry contributes 18% of the total greenhouse gas (GHG) emissions (Steinfeld et al., 2006). With the demand for livestock products set to double by 2050 (Steinfeld et al., 2006), it is essential that measures are taken to mitigate against unnecessary emissions in all aspects of livestock farming, including wastes. The numbers of breeding ewes in the UK approximates 14 million (DEFRA, 2012) and with an average mortality rate of 4% of adults and 10% of lambs (Bansback, 2006), a significant quantity of carcasses are generated that must be disposed of each year. Quantifying the GHG emissions due to livestock mortalities and their associated carbon footprint is therefore of interest.

Currently, most fallen stock in the EU is incinerated or rendered in accordance with the EU Animal By-Products Regulations, ABPR (Anon, 2009); and a low volume will be disposed of at hunt kennels or maggot farms (Kirby et al., 2010). Once an animal has died, it is stipulated that carcasses must be removed from the farm “*without undue delay*”, either by the farmer, the central collection service (a provision established by the UK Government in response to the introduction of the ABPR which was initially subsidised) or other waste licensed operators (Anon, 2009). Alternatively, fallen stock may be contained in airtight ‘Dolav’ containers for short-term storage (maximum of 7 days) during busy periods, and later collected by licensed waste collectors (WAG, 2011).

Novel methods of fallen stock containment can be used in the EU provided that they have been scientifically ratified and do not cause the proliferation of pathogens (Anon, 2009). Both bioreduction and freezing have been suggested as novel methods of storing carcasses. Freezing is quite simply the storage of carcasses in large-scale freezers on-farm prior to disposal so that numbers of carcasses can be bulked, making for more efficient collection. However, as a novel storage option it is considered to be cost-prohibitive due to the costs of freezing (Gwyther et al., 2012c). Bioreduction is a novel method for on-farm containment of dead livestock prior to disposal and is currently in the early stages of development. Animal carcasses and/or by-products are placed into a sealed vessel containing water, heated at 40 °C and aerated (50 kPa, 45 min h<sup>-1</sup>); resulting in the rapid degradation of carcasses into nutrient-rich liquor (Williams et al., 2009). Gases, primarily water vapour, are released to the environment through a vent containing an activated charcoal filter or biofilter, though some will also be emitted when opening the loading hatch (Williams et al., 2009). Although not yet approved for use with animal by-products in the EU, bioreduction has shown to be effective at reducing both the volume of waste (Williams et al., 2009) and bacterial and viral loads

(Gwyther et al., 2012b; Gwyther et al., 2012c). However, the environmental and social benefits of bioreduction have yet to be fully elucidated.

Carbon accounting can be used to assess both direct and indirect GHGs emissions from agricultural products and services or the farm as a whole, resulting in a carbon footprint (CF); a summary of the GHGs emitted (Carbon Trust, 2010; Edwards-Jones et al., 2009). The CF can then be used to pinpoint where improvements can be made to reduce GHG emissions, e.g. through reducing energy expenditure, or via the generation of renewable energy (Carbon Trust, 2010). Currently, carbon emissions from fallen stock are not included in whole-farm carbon accounting scenarios due to the lack of data (Edwards-Jones et al., 2009). This paper outlines the CF of bioreduction and compares it to the CF of existing methods of livestock storage and ultimate disposal, with the view of directing future research into the sustainability of bioreduction technology.

## **2. Materials and methods**

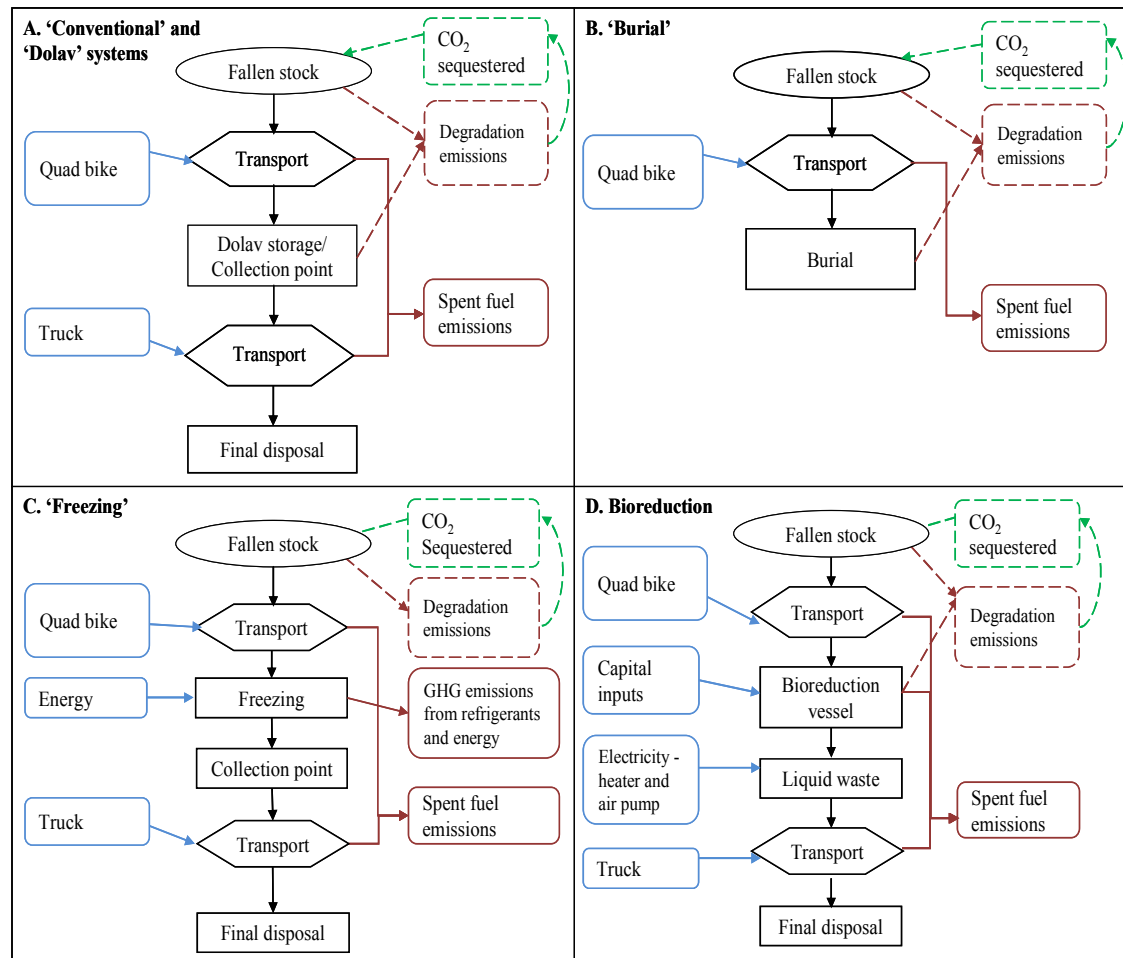
### *2.1. Functional unit*

The bioreduction of fallen stock was investigated by Williams et al. (2009) and forms the basis of the study (section 2.2.5), with the exception of the electricity data which was taken from a more recent trial (Gwyther et al., 2012a). Commercial-scale bioreduction vessels were sited at Bangor University's Henfaes Research Station, UK (53°14'05"N, 40°00'50"W); an active sheep farm with a breeding flock of 1,600 Welsh mountain ewes. During the year under investigation (2007-2008), the mortality rate was 5.5% of ewes; within the target range (5-10%) for Welsh sheep farms (HCCMPW, 2004). More specifically, over the year-long bioreduction study period, a total of 89 ewes and rams and 34 lamb carcasses were disposed of (Williams et al., 2009). The final weight of carcasses disposed was 2.82 tonnes which has therefore been chosen as the functional unit for this study.

### *2.2. System boundaries and assumptions*

Five storage scenarios for fallen stock were investigated in this study: collection of carcasses by the central collection service, hereafter referred to as the 'conventional' option; collection of carcasses using the 'Dolav' system; 'burial', 'bioreduction' and 'freezing'. Figure 1 shows the system boundaries of all fallen stock storage scenarios. In addition to the

GHG emissions released from these storage options, the end of life scenarios were also determined, namely; incineration, rendering and the potential use of bioreduction liquor as a fertiliser, along with variations in transport distances. All scenarios were based on a ‘death to disposal’ concept.



**Figure 1:** System boundaries for the four fallen stock storage options investigated. The quantity of CO<sub>2</sub> emitted during degradation was assumed to be equal to that sequestered in the fallen stock and therefore not reported (represented graphically by dashed arrows and outlines).

Conversion factors for energy and transport were obtained from standard databases such as Ecoinvent v2.0 for materials (Ecoinvent Centre, 2007) and the International Energy Agency figures for energy usage (IEA, 2010), and the CF of each scenario was carried out using the ISO14040 Life Cycle Assessment guidelines as a template (BSI, 1998; BSI, 2000a;

BSI, 2000b; BSI, 1997). In all cases, the CO<sub>2</sub> emissions arising directly from the final disposal of the fallen stock were assumed to be a replacement of sequestered CO<sub>2</sub> rather than an additional input of GHGs to the atmosphere. As such, any CO<sub>2</sub> from the decay of carcasses prior to and during collection was not included.

### *2.2.1. Conventional fallen stock storage*

Typically, once an animal dies on-farm it is collected by quad bike and taken to an accessible on-farm holding area before collection by a central collection service lorry, as part of a circular route that encompasses other farms. The fallen stock is then held briefly at a central depot until delivery to a final disposal unit. Collection distances were based on annual fuel consumption and carcass weight data supplied by a local waste collector (Anon, 2010b) and therefore considered representative of collections in North Wales. No “set up” impact has been included in this CF as it was assumed that no specialist equipment was needed.

### *2.2.2. ‘Dolav’ collection*

The main difference between the ‘conventional’ and the ‘Dolav’ system is the use of airtight ‘Dolav’ boxes, lined with a low-density polyethylene plastic liner. Each box has a capacity of 600 l, a tare weight of 37 kg (Dolav, 2012), is made of a low-density polyethylene and has a lifespan of 200 uses at 400 kg per use. A greater number of carcasses can be collected before disposal (maximum of 7 days post-death) due to the size of the box (WAG, 2011). As with the ‘conventional’ system, it was assumed that collection would be on a circular route or ‘milk-round’ basis with a new box delivered when the full box is removed. Also as for the ‘conventional’ system, the system modelled here is based on actual collection data from a local ‘Dolav’ collection agency situated in North West Wales, covering the counties of Anglesey and Gwynedd (Anon, 2010a).

### *2.2.3. Burial*

Although not currently legal in the majority of the EU, burial may be used in remote areas at the discretion of the competent authority (Anon, 2009). In addition, burial is also widely used as an acceptable form of livestock disposal in other countries outside of the EU. Yuan et al. (2012) qualitatively examined the emissions of CH<sub>4</sub> and CO<sub>2</sub> from the burial of

cattle carcasses in Nebraska, USA and determined that an average cattle carcass (500 kg live weight) produced 36 kg of CH<sub>4</sub> and 28 kg CO<sub>2</sub>. As sheep were used in the current study, it was assumed that the ratio of fat to muscle to carbohydrate were similar to those of cattle and that the GHG emissions would be proportionally comparable (Hui et al., 2001). As explained previously, the release of CO<sub>2</sub> from decomposition was considered biogenic and not included in the carbon account. As for all scenarios, a collection distance of 200 m by quad bike was used to represent the removal of animal carcasses to the burial site. It was assumed that carcasses would be buried as and when they died. The final assumption was made that no specialist equipment would be required to bury the carcasses as it could be undertaken using standard farming equipment (i.e. tractor).

#### *2.2.4. Freezing*

Though not a common practice, the on-farm freezing of fallen stock was studied here as it may allow for: 1) less frequent and more efficient transport of fallen stock than conventional collection, and 2) a larger quantity of solid material to be rendered than that produced by bioreduction. The same assumptions were made as for 'conventional' collection with the exception that the transport emission factor was the same as for bioreduction; modelling a relatively efficient and full truck load. The freezer size required for a functional unit of 2.82 t of fallen stock over one-year was estimated at being a 3 m<sup>3</sup> walk-in freezer. Emissions from freezing were calculated from an existing life cycle assessment (LCA) produced by LCADK (Nielsen, 2002).

#### *2.2.5. Bioreduction*

Prior to the on-farm trial, the fibreglass/resin bioreduction vessels were transported from Barcelona, Spain to the UK by means of truck and ferry. A JCB digger was used to prepare the hole in which the vessels were situated, taking half a day per vessel. Once established, the bioreduction process entailed the following stages: the fallen stock were collected from the field as normal (section 2.2.1); placed into the vessel along with an initial 2,800 l of water; and the vessels operated according to the manufacturer's instructions, i.e. aerated at a pressure of 50 kPa for 45 min every hour and heated by means of an oil-filled electric heating element running the length of the vessel which was regulated by a thermostat. For every kg of carcass, 1 g of a commercial 'Ingester Product' was added to the vessel. This

is a product derived from seaweed (*Ascophyllum nodosum*) and is meant to facilitate bioreduction by serving as a substrate and a nutrient source for microbes as well as accelerating autolysis of cells in the fluid phase (Gutiérrez et al., 2003). Additional water (400 l) was added to ensure that  $\frac{2}{3}$  of the carcasses were covered at all times, as recommended by previous studies (Gutiérrez et al., 2003). All fallen stock produced from the flock of 1,600 ewes were successfully bioreduced in one vessel (Williams et al., 2009). At the end of the trial, the tank was drained by means of a liquid waste disposal truck and transported 54 km to a rendering plant for ultimate disposal (2.5 t). Additional waste collections were made but only the impacts allocated to bioreduction were included in this study. The impact of the production and instalment of the bioreduction vessels was allocated over an assumed 25 year lifetime, as per the manufacturer's guarantee. Primary data was collected for the bioreduction scenario only and consisted of GHG emissions and transport distances (Williams et al., 2009).

### *2.3. End of life scenarios*

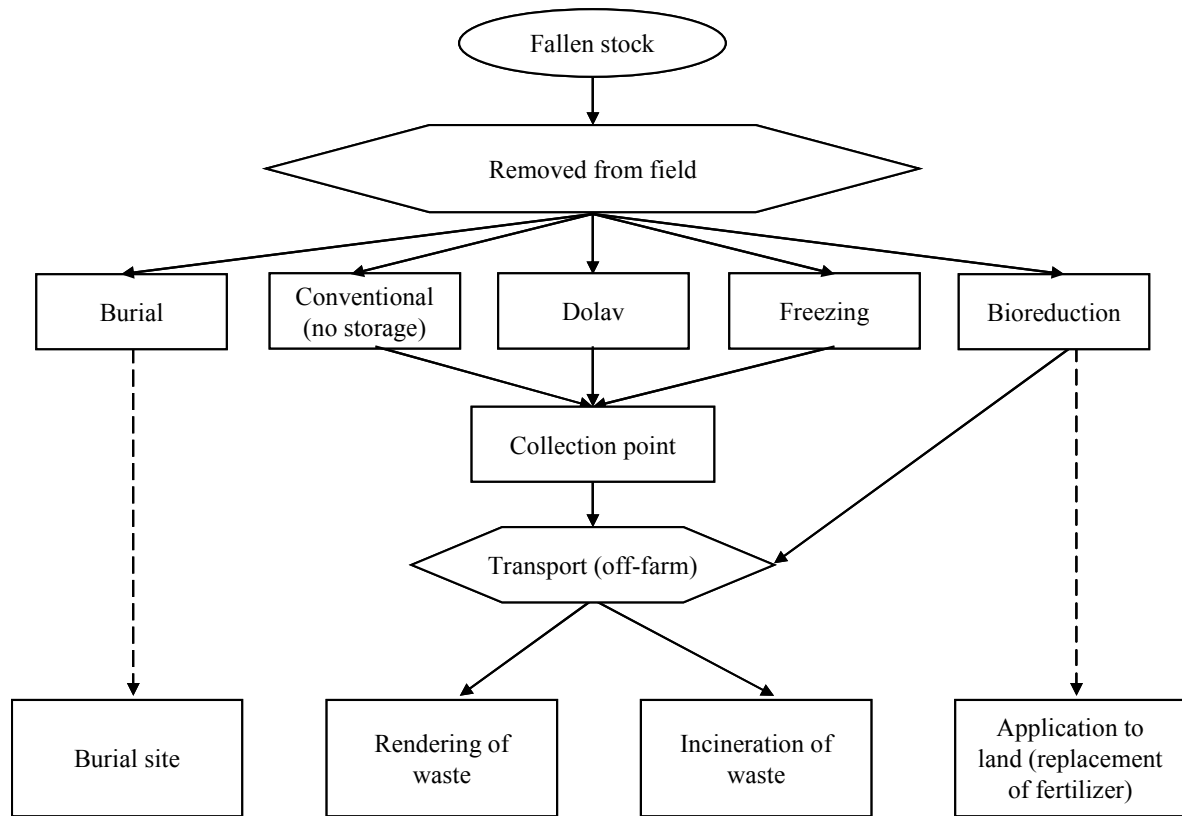
For a more complete picture of the impact associated with each collection method, the end of life impact was also calculated. Under the current EU ABPR, the disposal options available for sheep carcasses, which are classified as Category 1 (high risk) due to the potential risk of transmissible spongiform encephalopathy (TSE) agents in specified risk material, are limited to rendering and incineration (Anon, 2009). Whilst the liquor waste generated for bioreduction would likely be incinerated as part of a mixture of general liquid waste (Woodgate, 2011), the application of the waste to land as a fertilizer replacement may theoretically be an option if it were to be proven safe. The options studied in this report are shown in Figure 2.

#### *2.3.1 Incineration*

Incineration in this context was described as the combustion of organic substances contained in waste materials. In this case the organic matter present (carcasses) does not (alone) fuel the combustion and as such there was a need to include an additional fuel source, as opposed to a simple burning process. Data for the option of incineration was gathered from the local collector for the year 2007 where they provided an incineration service for cattle during the foot and mouth outbreak (Anon, 2010b). The impact of incinerating carcasses



(where the biogenic release of sequestered CO<sub>2</sub> is assumed to balance sequestration and not included) was calculated from average natural gas usage in 2007 as 1,563 kWh per tonne of incinerated matter and 0.185 kg CO<sub>2</sub> e per kWh of natural gas used (Carbon Trust, 2008).



**Figure 2:** End of life options for each of the 5 storage/collection scenarios: ‘burial’; ‘conventional’; ‘Dolav’, ‘freezing’ and ‘bioreduction’. Dashed arrows represent on-farm transport.

### 2.3.2. Rendering

Rendering is a process that converts waste animal tissue into stable, value-added materials such as rendered fats (e.g. tallow) and processed animal protein (PAP). Rendering consists of both physical and chemical transformations through heating and separation of fallen stock and slaughterhouse by-products, as well as butchery and supermarket waste. There are two main systems of rendering, described as either wet or dry systems depending on whether steam is added or water is evaporated off respectively, with the latter being further divided into natural fat and added fat systems (Woodgate and van der Veen, 2004). The assessment of the environmental impact (GHG emissions) of this industry is complex

due to the allocation issues surrounding the quantity of tallow used as a fuel as well as it being sold as a by-product. Further difficulties arise in finding an average as different rendering plants also deal with different categories of animal by-products and as such they will either be burnt as fuels (waste classified as Category 1 and 2), or used in the fertiliser, pet food and chemical industries (waste classified as Category 3) as per the ABPR (Anon, 2009). Data regarding the rendering industry was however kindly supplied by Harper Adams University College and the European Fat Processors and Renderers Association (Ramirez et al., 2010; Ramirez et al., 2012). An average of 40.8% of tallow produced was assumed to be co-combusted, therefore reducing the carbon account of the rendering plant (Ramirez et al., 2010). An in-depth discussion of the final assumptions made can be found in Norton et al. (2011).

### *2.3.3. Application to land*

In the farm trial described by Williams et al. (2009), the resulting bioreduction liquor waste was sent for incineration with other liquid wastes. However, if bioreduction was shown to eliminate all pathogens then this may deem it suitable for disposal onto land; especially from bioreduction vessels containing pigs or poultry as such waste will not contain specified risk material and therefore are classed as Category 2 wastes under the EU ABPR. By observing the nutrient value of the liquor waste (Williams et al., 2009), it can be calculated how much synthetic fertiliser use could be offset if the waste was applied to land and the associated savings in greenhouse gas emissions. Emission values for equivalent synthetic N, P and K fertilizers were calculated as an average from those available in the Ecoinvent v2 library (Ecoinvent Centre, 2007).

### *2.4. Seasonal variation and transport distances*

Seasonality data was provided by Anon (2010b) and included two full years of data on fuel use and fallen stock weight for 2007 and 2008, with partial data for 2006 and 2009. In order to study the impact of varied transportation distances, the tkm (tonne weight multiplied by km distance) impacts for the farm study were extrapolated with the assumption that the impact would be the same proportionately over varied transport distances. The distance used here is the most direct distance from the farm to final destination using roads, not the total distance travelled by the truck as this would vary greatly due to the complex nature of the

collection routes. Collection depots were assumed to be at the half-way point between the farm and final disposal.

## 2.5. Renewable energy sensitivity analysis

Data from a commercial company was used to compare the cost of using renewable energy against taking electricity from the national grid (EvoEnergy, 2012). Electricity readings from a recent bioreduction study using pig carcasses were used to estimate energy usage for a one-year period (Gwyther et al., 2012a). Inflation was taken as being the average inflation rate for the years 2001 – 2011 (Bank of England, 2012) and the cost of a kWh of electricity was taken from the Energy Saving Trust (Energy Saving Trust, 2012).

**Table 1:** Summary of all emissions in kg CO<sub>2</sub>e associated with each collection phase and end of life scenarios (shown here in italics). End of life emissions take into account emissions produced during the collection phase and are therefore not in addition to, but inclusive of emissions from the collection phase. N/A indicates not applicable.

System	Collection phase	<i>If incinerated</i>	<i>If rendered</i>	<i>If used as fertilizer</i>
Conventional	194	997	406	N/A
Dolav	155	958	367	N/A
Burial	4254	N/A	N/A	N/A
Freezing	3758	4561	3970	N/A
Bioreduction	10139	10850	10299	10116

## 3. Results and discussion

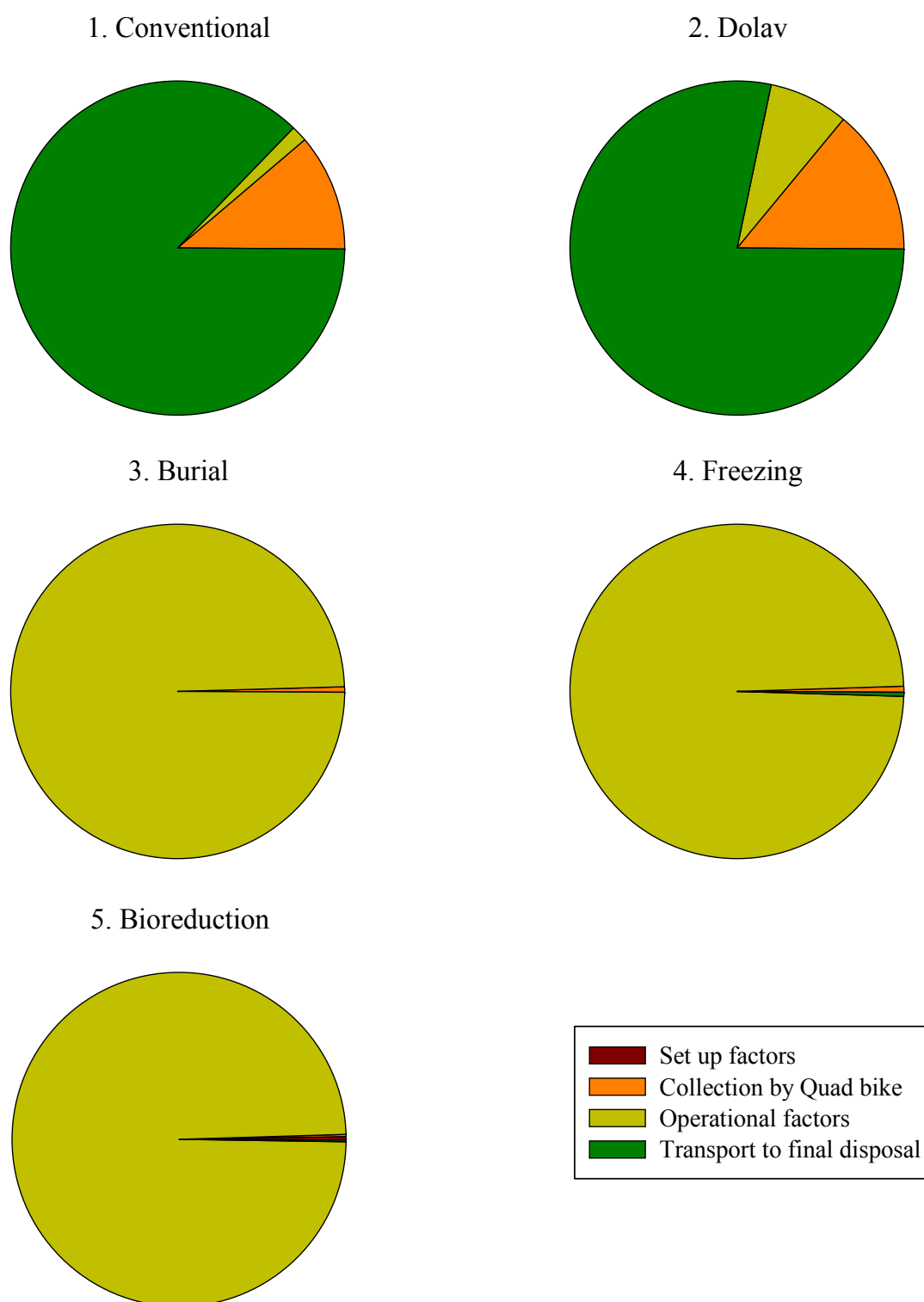
### 3.1. Conventional and Dolav collections

The total annual impacts associated with each fallen stock storage scenario and the associated end of life options can be found in Table 1. Transport to final disposal contributed the highest impact for both the ‘conventional’ and ‘Dolav’ collection systems (Figure 3), whereas, for ‘bioreduction’ and ‘freezing’, the electricity used during the operational phase produced the most CO<sub>2</sub>e. Methane produced during the decomposition of carcasses

contributed the highest impact for burial. Both the ‘conventional’ and ‘Dolav’ collection scenarios modelled here were based on annual diesel and carcass collection data from a local waste collector (Anon, 2010b) and so, represent the situation in North Wales. However, despite having relatively lower CF than all other options (194 and 155 kg CO<sub>2</sub>e respectively) there is the potential for increased biosecurity risks associated with central collection services. If, during the bio-reduction trial the carcasses had been removed by the ‘conventional’ method, 40 trips would have been needed to remove the carcasses in strict accordance with the legislation. For the ‘Dolav’ collection method, it would have been 22 and, in the majority of cases, the Dolav bins would have been near empty (Williams, 2012). Given the concerns within both the UK and Welsh industry with regards to the transport of carcasses after the foot and mouth disease (FMD) outbreak of 2001, where transport of diseased animals played an import role in the spread of the disease in the UK, alternative on-farm options are gaining favour (Bansback, 2006). Additional concerns arise from the logistical problems associated with the collection of fallen stock from rural and upland areas such as those that cover much of Wales, particularly during periods of high mortality, in the allotted timescales dictated by the legislation (Bansback, 2006).

### *3.2. Burial*

Burial was a widespread practice prior to introduction of the EU ABPR and is currently allowed in ‘remote’ areas of the EU and therefore has been included in this study for comparison. Burial had an annual impact of 4,254 kg CO<sub>2</sub>e due to the direct emissions of CH<sub>4</sub> released during the decomposition of the carcasses. Caution should be taken when interpreting these results as the emissions released were based on figures for cattle, and those were inferred by the authors from the anaerobic decomposition of carcass material in water and only took into account burial in water-logged soils (Yuan et al., 2012). In addition, no account was made for the conversion of CH<sub>4</sub> to CO<sub>2</sub> due to oxidation in soils (Yuan et al., 2012), or for the scavenging of carcass material by animals. Unfortunately, without directly determining the GHG emissions from carcass burial, it is inevitable that such assumptions must be made. In addition, the release of GHGs during the breakdown of carcasses prior to or during collection was not included in this study for the other scenarios and consequently, these other options may be slightly underestimated. Nevertheless, the results do indicate that there is the potential for a significant release of GHGs due to the anaerobic nature of below-ground burial in waterlogged sites.



**Figure 3:** The proportion of CO<sub>2</sub>e emitted attributed to each stage in the lifecycle of carcass disposal

### 3.3. Freezing

Freezing suffered from a high CF associated with the consumption of electricity. Indeed, electricity usage accounted for 99.9% of the CO<sub>2</sub>e released (or 3,721 kg CO<sub>2</sub>e). This data lends support to the theory that, given such a high electricity usage for ‘freezing’, it is unlikely that this will be a favourable livestock storage option within the agricultural community due to the associated energy costs (Gwyther et al., 2011).

### 3.4. Bioreduction

As the technology stands, the CF of bioreduction does not fare favourably with existing collection and disposal strategies (Table 1). Bioreduction has a CF 2.3 times higher than burial, the disposal option with the next highest CF (10,139 kg CO<sub>2</sub>e and 4,254 kg CO<sub>2</sub>e respectively), 2.7 times higher than freezing, and 52 and 65 times higher than for the ‘conventional’ and ‘Dolav’ collection services, respectively. Electricity data was extrapolated from a trial using 140 kg pig carcasses over a two month period (Gwyther et al., 2012a). In this instance, electricity data was recorded five days prior to the addition of carcasses and throughout the two month trial, and showed little fluctuation over time (linear relationship  $R^2 = 0.999$ ). This indicated that the majority of electricity was used to heat the water rather than the carcasses (Gwyther et al., 2012a). Therefore, the electricity data was extrapolated to twelve months and used in this trial, regardless of the number of carcasses added. The principal reason for the high emissions stem from the vast consumption of electricity used to heat the water to 40 °C (10,068 kg CO<sub>2</sub>e). There are several ways in which the reliance on electricity can be reduced. The simplest solution would be to simply switch off the vessels during periods of low mortality. Although decomposition would be retarded, any pathogens would still be contained within the vessel, thus maintaining biosecurity (Gwyther et al., 2012c). Another solution would be to lower the operating temperature. Again, this may reduce the decomposition rate, thus necessitating a higher frequency of vessel emptying, or a proportion of carcasses to be collected by either the ‘conventional’ or ‘Dolav’ systems, to compensate for the higher volume of waste still in the bioreduction vessels. There is also the potential for reduced aeration; however, this may lead to more anoxic conditions within the vessels and the subsequent production of methane which has a higher global warming potential. Further experimentation is needed to determine if any of these solutions are feasible without impairing biosecurity or ease of operation.

Reducing the volume of water needed in the process may prove an effective method of minimising electricity usage as this is where the majority of electricity consumption occurs. It would also make the technology more appealing in those areas where water is not immediately accessible. However, experience with the current vessel design indicates that using less water can lead to problems with the oil filament overheating and shorting the electricity circuits (Williams et al., 2008). Current vessel design flaws stem from the fact that the vessels were originally designed for a warmer Mediterranean climate (Gutiérrez et al., 2003) where less energy would have been needed to heat the water. Therefore, bioreduction in its current form may not have such a high CF in countries with a high mean annual temperature. In temperate climates, insulation of the bioreduction vessels is essential, both above and below ground level. Currently, the vessels are made of a single layer of thermostable fibreglass and chemically resistant resin (Gutiérrez et al., 2003). It is recommended that future vessels are designed using a double layer of fibreglass and resin with an insulating layer in the centre. Whilst this would increase the proportion of CO<sub>2</sub>e associated with the set-up of the vessels, it would be allocated over the 25 year lifespan of the vessel increasing the U value of the bioreducer.

From an economics perspective, it would indeed be cheaper to run the bioreduction vessels using photovoltaic energy; £66,000 using photovoltaic energy compared to £70,700-£118,800 using conventional electricity usage over the 25 year lifespan of the vessels. Nevertheless, it is estimated that it would take 130 m<sup>2</sup> of solar panels to produce sufficient electricity to run a bioreduction vessel using photovoltaic energy (EvoEnergy, 2012). Therefore, the large initial expenditure and the amount of land required to site photovoltaic panels make this option impractical for the farming community. Renewable energy is not therefore considered to be a suitable mitigation method although it would indeed reduce the CF. Therefore, in order to significantly reduce the CF of bioreduction it would take a complete overhaul of the fundamental design of the system; namely, changing the vessel design from heating using electricity to heating using a boiler.

### *3.5. End of life scenarios*

The additional impacts derived from the end of life scenarios can also be found in Table 1. The rendering of carcasses collected through the 'Dolav' system had the lowest overall impact (367 kg CO<sub>2</sub>e) whereas the highest impact is from bioreduction followed by

incineration (10,850 CO<sub>2</sub>e). If the liquor from bioreduction could be used as a fertiliser on-farm it would effectively negate the impact of final disposal and would reduce chemical fertiliser usage. Therefore, total N replaced would equate to a saving of 19.5 kg CO<sub>2</sub>e; for total P<sub>2</sub>O<sub>5</sub>, 1.53 kg CO<sub>2</sub>e; and for total K<sub>2</sub>O, 1.2 kg CO<sub>2</sub>e (Ecoinvent Centre, 2007). However, this impact is very small when compared to the total impact of the collection phase (Table 1). Unless TSEs are shown to be removed entirely during the bioreduction of sheep carcasses, it is highly unlikely that liquor from the bioreduction of sheep will be approved for use as a fertiliser. However, given that there are no reported cases of TSEs in either pigs or poultry, liquors from these animals may be considered suitable for land-disposal in the future.

### *3.6. Seasonality and transport distances*

Seasonal variation exists in the ‘conventional’ collection of fallen stock due to the increased transport efficiency during the lambing season, when greatest losses occur on sheep farms. Outside of the lambing period, an average of 67 l of diesel was used per tonne of fallen stock collected; during lambing time this dropped to 30 l. As an average for the year, 42 l of fuel was used per tonne collected; this was used as the basis for the tkm calculations for all transport calculations. Due to the complexity of a ‘milk round’ style collection method used by both the ‘Dolav’ and ‘conventional’ style of collection it is likely that the tkm impact used here may not always be entirely reflective of actual values. The data should therefore be regarded as an approximation of the relative effects of varied transport distances on CFs of the studied systems (Table 2). It is perhaps surprising to see that the Dolav collection method has such a high impact factor for the transport of carcasses to the depot (0.778 kg CO<sub>2</sub>e), but a contributing factor in this case is likely to be the limiting number of Dolavs that are able to fit on the collection truck at one time which will in effect limit the quantity of fallen stock that can be collected thus reducing efficiency.

As can be seen from Figure 4, the increase in CF for the ‘conventional’ and ‘Dolav’ methods of disposal is greater with increased transport distances. This is due to the lower proportion of impacts associated with transport for the ‘burial’, ‘freezing’ or ‘bioreduction’ scenarios. At no point does the impact associated with bioreduction intersect with either the ‘conventional’ or ‘Dolav’ system, not even after 1,000 km (Fig. 4). According to the UK Renderers’ Association, the ‘reasonable’ transport distance to rendering facilities is 200 km



by road (Ramirez et al., 2012). Therefore, bioreduction would not seem to be a favourable option.

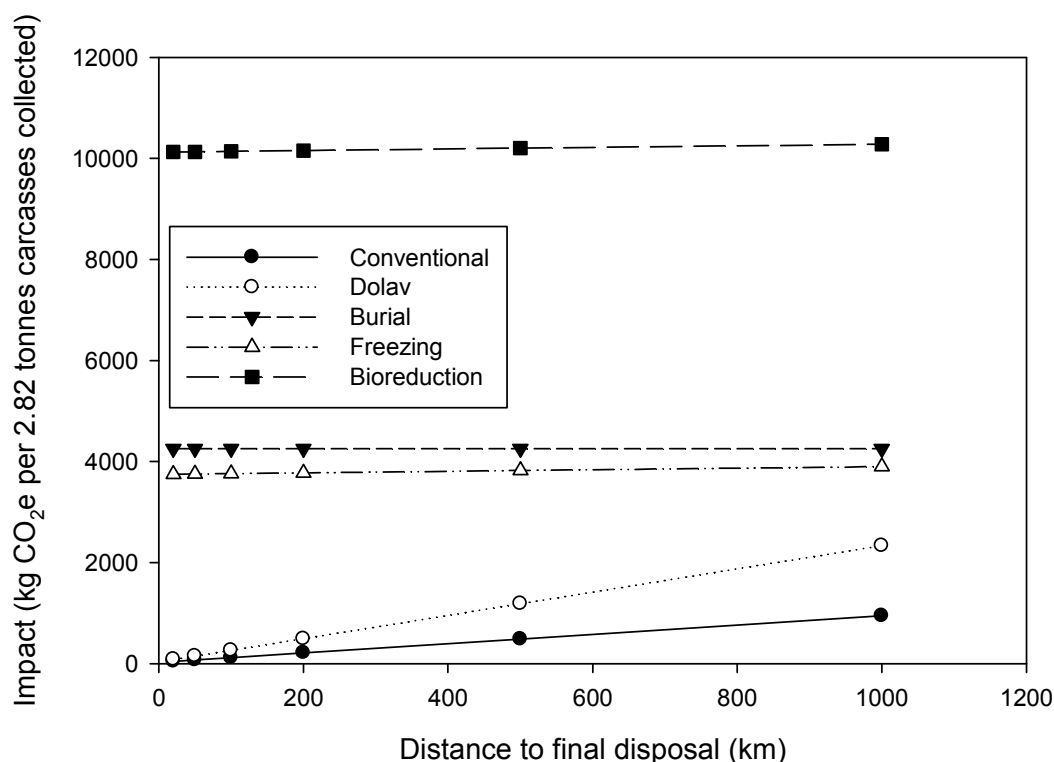
**Table 2:** Summary of all tkm impacts for each of the fallen stock scenarios. N/A indicates not applicable.

System	Transport stage	Distance (km)	Impact per tkm (kg CO <sub>2</sub> e)
Burial	N/A	N/A	N/A
Conventional	impact to depot *	100.0	0.286
	impact to renderer	46.0	0.041
Dolav	impact to depot *	20.0	0.778
	impact to renderer	128.7	0.038
Freezing	impact to renderer	109.4	0.027
Bioreduction	impact to renderer	109.4	0.027

\* calculated as an average farm to collection depot, as established with the collection agency

### 3.7. Livestock disposal in the context of whole farming

Whilst the figures presented in this study enable a comparison of carbon footprints for different carcass storage and disposal routes, they should be considered in the context of emissions from livestock farming as a whole. It can be shown that even an inefficient method such as freezing fallen stock still provides a relatively minor impact when compared to the emissions from livestock production as a whole. This is mainly due, but not limited to, the emission of CH<sub>4</sub> from the ruminant stock on a typical Welsh sheep farm producing an average of 12.9 kg CO<sub>2</sub>e per kg live weight of sheep (Edwards-Jones et al., 2009); which in this example farm would equate to 619 tonnes of CO<sub>2</sub>e per year from the flock of 1,600 breeding ewes (assuming a live weight of 30 kg each) at the case-study farm. As such, even employing the ‘bioreduction’ method would theoretically equate to just 1.6% of the flock’s total CF. It should also be noted that the simplicity of the bioreduction livestock storage system makes it an attractive option for livestock farmers which may have the benefit of increased compliance with the Animal By-Products Regulations. Whilst the case study site is typical of an upland Welsh sheep farm, this study makes no attempt to extrapolate the findings to the sheep industry in other parts of the world.



**Figure 4:** The total impact for each disposal scenario taking into account the impact associated with varying transport distances (km).

### 3.8. The future of bioreduction

Whilst the CF of bioreduction highlights the need for improvements with regards to energy efficiency, other factors should also be considered when assessing the environmental credentials of the system. For example, a reduction in transport could lead to a reduction in particulates and nitrous oxide emissions from vehicles. Using bioreduction liquor from the pig and poultry industry could potentially replace chemical fertiliser usage although further studies are required to determine the leaching of nutrients associated with a liquid fertiliser application (section 3.5). Finally, storing carcasses in an enclosed (and potentially double-layered) vessel will prevent the spread of zoonotic agents potentially arising from the burial of carcasses to the water supply (see Gwyther et al., 2011 for a review of carcass burial). This is of particular importance to those areas designated as ‘remote’ according to the ABPR (Anon, 2009) where burial is still an acceptable method of livestock disposal. In these cases, the risk of burying diseased carcasses may outweigh the impact of GHG released during the bioreduction process.

#### **4. Conclusions**

Bioreduction has the highest carbon footprint of all the disposal methods assessed in this study. Without completely redesigning the vessels to reduce the reliance on electricity, it is unlikely that the impact of bioreduction will be significantly reduced. Nevertheless, in the context of whole farming, bioreduction of fallen stock would lead to a minimal increase in a farm's overall CF. In addition, the benefits of improving biosecurity by limiting transport of carcasses between farms may well outweigh the negative impacts of the carbon footprint.

#### **Acknowledgements**

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## **Characterisation of physicochemical cycling and enzyme activity during the bioreduction of pig carcasses**

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## ABSTRACT

Bioreduction is a novel method for the on-farm storage of fallen stock in a vessel containing water that is heated and aerated, prior to disposal. The combination of a mesophilic temperature and high bacterial population leads to rapid degradation of carcasses due to microbial and enzymatic breakdown of protein material; and ultimately the reduction in volume of waste to be disposed. Understanding the physicochemical cycles and enzyme activity within bioreduction vessels could direct future enhancements of the system. Two commercial-scale bioreduction vessels (6.5 m<sup>3</sup> capacity) were fed with pig carcasses and changes in physicochemical parameters, enzyme activity, gas emissions and microbial communities were analysed over 56 days. Physicochemical parameters tested showed different properties depending on the vessel, with microbial communities in both vessels converging between days 28 and 42 before once more diverging. Of all enzymes assayed, acetylsterases showed the highest activity at the start of the bioreduction process with a subsequent increase in lipase towards the end. All other enzymes showed little activity in comparison. Despite active aeration of the vessels, conditions were redox-constrained leading to the emissions of some gases associated with anaerobic conditions; namely CO<sub>2</sub>, NH<sub>3</sub> and H<sub>2</sub>S. It was concluded that no single parameter directed the biochemical processes and that each bioreduction vessel will have its own unique microbial population and rate of decomposition. However, there is the potential for further efficiencies potentially using bioaugmentation or enzyme additives.

*Keywords:* agriculture; anaerobic digestion; decomposition; fallen stock; waste

## 1. Introduction

The introduction of the Animal By-Products Regulations, ABPR, in 2002 prohibited traditional on-farm methods of fallen stock disposal; i.e. burial and pyre burning (Anon, 2009). This has led to consternation within the agricultural community due to biosecurity concerns related to the transport of carcasses (Bansback, 2006). However, novel methods of fallen stock disposal or storage can be proposed under the ABPR (Anon, 2009). Bioreduction is one such novel on-farm carcass *storage* system that has previously been shown to be efficient at reducing the volume of waste that ultimately needs disposing (Williams et al., 2009). It is defined as the “aerobic biodegradation of animal by-products in a partially sealed vessel [containing water], where the contents are mildly heated and aerated” (Williams et al., 2009). Further enhancements in efficiency would allow for a higher volume of carcasses to be decomposed which has both practical and economic benefits for the farmer. To optimise bioreduction, the processes involved must be understood. Whilst some elucidation of the biochemical nature of the liquor (liquid waste) has been done (Williams et al., 2009), this study sought to assess temporal changes in enzyme activity and microbial communities as both factors directly drive the nutrient cycles in this almost completely enclosed system.

Enzymes are the cornerstone to successful bioreduction as they facilitate the degradation of the animal carcass into lower molecular weight molecules which can be assimilated by microorganisms, thus ensuring the continuation of the microbial community. Provenance of intrinsic carcass decomposition enzymes has been difficult to elucidate and indeed very little is reported in the literature about specific enzymes involved in the decomposition process from a carcass itself. However, decomposition enzymes will be involved at every stage and pathway of bioreduction; deriving from the carcass tissue during autolysis (Evans, 1963; Vaas, 2001), from internal microorganisms during putrefaction (Evans, 1963; Micozzi, 1991) as well as to a lesser extent from external sources (Carter et al., 2010; Howard et al., 2010; Putman, 1978). Whilst it is out of the remit of this study to determine the source of the enzymes found in the liquor, it is possible to determine the relative activity of each of the major enzyme families thought to be involved i.e. proteases, esterases, lipases, aryl sulphatases,  $\beta$ -D-glucosidase, and phosphatases (Parkinson, 2009), whilst using the enzyme dehydrogenase as a measure of overall microbial activity.

A side-effect of this decomposition of organic material is the production of gases. Williams et al. (2009) analysed several gases released during the bioreduction of sheep carcasses ( $\text{CO}_2$ , CO,  $\text{O}_2$ ,  $\text{NH}_3$ ,  $\text{H}_2\text{S}$  and  $\text{CH}_4$ ) and found no significant differences between the

composition of gases released from the bioreduction vessels with that of ambient air. Odours on the other hand were noted approximately 200 m downwind of the bioreduction vessels and were particularly potent when the loading hatches were opened during the first two weeks of bioreduction. Gas emissions can reveal the mechanisms behind the nutrient cycling within the bioreduction vessels. Therefore, in order to build a wider picture of the nutrient cycling within the bioreduction vessels gases and odour have been determined in this study.

In order to test the hypothesis that microbial community changes are directly linked to the enzyme activity changes in the liquor, this study combines concurrent analysis of microbial communities using automated ribosomal intergenic spacer analysis (ARISA) combined with enzyme activity, physicochemical factors and gas emissions in order to facilitate the observation of community and microbial activity changes over time. Knowledge of specific enzymes and their activities along with microbial communities involved in decomposition are pivotal for the development of a starter inoculum that will improve efficiency and thus the economic viability of bioreduction.

## **2. Methods**

### *2.1. Trial management*

Two commercial scale bioreduction vessels (BVs) located at Bangor University's Henfaes Research Station, Wales, UK (53°14'05"N, 4°00'50"W) were used in this trial (Williams et al., 2009). Pig carcasses ( $140 \pm 5$  kg) were placed in each vessel containing 3,100 L of liquor from a previous bioreduction process and run under standard conditions (40 °C, aerated at a pressure of 50 kPa 45 min<sup>-1</sup>) (Gwyther et al., 2012; Williams et al., 2009) for two months with no further carcass additions. BVs were connected to a woodchip/compost biofilter bed (1 × 2 × 3 m; h × w × d) by the use of a central pipe.

### *2.2. Liquor sample collection*

At all sampling times, three samples of the liquor were taken (250 ml) from each vessel. Samples were taken on day 0, 7, 15, 21, 28, 42, and 56. Liquor depth was recorded for each vessel using a graded sampling pole. Electricity readings were taken from an on-site electricity meter.

### 2.3. Physicochemical analysis

Temperature was measured in the field. Dissolved oxygen (DO), pH, turbidity and electrical conductivity (EC) were measured on return to the lab using a Hanna HI 9142 Dissolved Oxygen Meter (HANNA instruments, Smithfield, USA); Martini Mi 150 benchtop pH meter (Milwaukee Instruments Inc, Menomonee Falls, USA); Eutech T-100 turbidity meter (Eutech Instruments, Singapore); and Hanna HI 9033 multi-range conductivity meter (HANNA instruments, Smithfield, USA) respectively. Turbidity and EC samples were diluted with distilled water in either a 1:2 or 1:3 (v:v) dilution and the results amended accordingly.

Moisture content and organic matter analysis were analysed on 5 ml sub-samples using standard methods, i.e. drying at 105 °C for 24 hours and ashing at 450 °C for 24 hours, respectively. All further analyses were treated according to the procedure of Williams et al. (2009) with an additional filtering step (Whatman No 1). Total carbon and total nitrogen were analysed using a TC-TNV analyser (Shimadzu Corp., Kyoto, Japan).  $\text{NO}_3^-$  and  $\text{NH}_4^+$  were determined using a PowerWave XS spectrophotometer (BioTek Instruments Inc, Winooski, USA) using standard methods (Miranda et al., 2001; Mulvaney, 1996) whilst  $\text{PO}_4^{3-}$  was analysed based on the methods of Murphy and Riley (1962). The Bradford method was used to analyse protein content (Bradford, 1976) using a commercial Bradford Reagent (Sigma-Aldrich Company Ltd., Dorset, UK) whilst carbohydrates were analysed according to Safarik and Santruckova (1992).

### 2.4. Enzyme analysis

Substrate saturation and optimal incubation tests were elucidated using laboratory-scale bio-reduction with cubed pork pieces as the substrate (data not shown). All enzyme substrates and chemicals were obtained from Sigma-Aldrich Company Ltd. (Dorset, UK) unless stated otherwise. All activities are expressed as the mg substrate released  $\text{l}^{-1}$  liquor  $\text{min}^{-1}$  due to the high moisture content of the liquor.

#### 2.4.1. Protease activity

The Fluoro<sup>TM</sup> Protease Assay kit was used according to manufacturer's instructions (G-Biosciences, St-Louis, USA).

#### 2.4.2. Hydrolase activity

The hydrolase assays were based on the procedure of Freeman et al. (1995). MUF substrates were dissolved in 2 ml methoxy ethanol (Methyl cellosolve ®) before being made up to 50 ml with distilled water (see Table 1 for final concentrations). Substrate (7 ml) was mixed with liquor (1 ml) by gentle inversion and incubated according to Table 1. Liquor-substrate suspensions (1.5 ml) were added to micro-centrifuge tubes and centrifuged for 5 min at 10,000 rev min<sup>-1</sup>. Supernatants (300 µl) were added to the wells of a black 96 well plate. Fluorescence was measured using a Varian Cary Eclipse spectrophotometer (Varian Inc., Palo Alto, USA) (330 nm excitation and 450 nm emissions, slit setting 2.5 nm) and corrected for background fluorescence using a control blank.

**Table 1:** The name and classification of hydrolase enzymes assayed and the corresponding 4-methylumbelliferyl (MUF) fluorescent substrate

Enzyme	Enzyme code	Substrate	Substrate supplier	Substrate concentration (µM)	Incubation period (min)
Acetylesterase	EC 3.1.1.6	MUF-acetate	Glycosynth	400	20
Acetylesterase	EC 3.1.1.6	MUF-butyrate	Sigma	400	20
Lipase	EC 3.1.1.3	MUF-nonanoate	Glycosynth	400	60
Phosphatase	EC 3.1.3.2	MUF-phosphate	Sigma	900	20
Arylsulphatase	EC3.1.6.1	MUF-sulphate potassium salt	Sigma	900	60
β-D-glucosidase	EC 3.2.1.21	MUF-β-D-glucopyranoside	Glycosynth	600	60

#### 2.5. Gas analysis

Gas analysis in the field was undertaken using a handheld gas meter (GFM416 Series Gas Analyser, Gas Data Ltd., Coventry, UK) which analysed CH<sub>4</sub>, CO<sub>2</sub>, O<sub>2</sub>, H<sub>2</sub>S, and NH<sub>3</sub>. Gas readings were taken from the outlet pipe of each bioreduction vessel, the surface of the biofilter and 10 m upwind and downwind of the bioreduction vessels. All gas readings were taken in triplicate.

## 2.6. *Odour analysis*

The odour intensity scale used by Jiang et al. (2006) was used to assign values to the odour emanating from both the sealed, and opened, bioreduction vessels on the day of sample collection.

## 2.7. *Microbiological characterisation*

### 2.7.1. *Dehydrogenase activity*

Dehydrogenase activity (DA) was determined according to standard composting procedures, where 3 g of compost was replaced with 3 ml of liquor (US Department of Agriculture and US Composting Council, 2001). Values were corrected for background absorbance using control blanks.

### 2.7.2. *Bacterial total viable counts*

Liquor (25 ml) was homogenised with 225 ml of maximum recovery diluent (MRD, CM0733; Oxoid Ltd., Basingstoke, UK) in a Seward 400 Stomacher (Seward Ltd., Worthing, UK) for 1 min at 230 rev min<sup>-1</sup>. The homogenate was serially diluted in MRD before plating onto R2A agar (CM0906; Oxoid Ltd., Basingstoke, UK). Plates were incubated at 20 °C for 7 d before enumeration.

### 2.7.3. *Bacterial community analysis using ARISA*

Frozen liquor stocks were defrosted at 4 °C for 48 hours before DNA was extracted according to a modified protocol of Anderson and McKay (1983) (Table 2) and visualised on an 0.8% agarose gel stained with SafeView Nucleic Acid Stain (NBS Biologicals, Huntingdon, UK). PCR was performed using the primer set 132f/1522r (Ranjard et al., 2001) with each 10 µl reaction containing 4.3 µl H<sub>2</sub>O, 2 µl of a PCR-enhancing mixture (3 M betaine and 1% Tween 20), 1 µl MgCl<sub>2</sub>, 0.5 µl of 5 mM mixed dNTP (Promega, Southampton, UK), 0.5 µl of 10 pmol of each primer (Eurofins MWG Operon, Ebersberg, Germany), 0.2 U Taq DNA polymerase (Roboklon, Berlin, Germany), and 1 µl 10x PCR buffer B (Roboklon, Berlin, Germany). DNA was diluted to a concentration of 10-50 ng µl<sup>-1</sup> and 1 µl was added to each reaction. The intergenic spacer region between the 16S and 23S

rRNA genes was PCR amplified according to Gertler et al. (2012). ARISA profiles were produced using an Agilent DNA 1000 kit with the Agilent 2100 Bioanalyser (Agilent, Wokingham, UK) following the manufacturer's instructions and default settings for the DNA 1000 chip. The internal length standard of the DNA 1000 kit was used to normalise band patterns automatically. A band-matching analysis was performed using the square root function of peak height. Similarity of fingerprints were analysed using the Bray-Curtis model and nonmetric multidimensional scaling (MDS) using the PAST program (Hammer et al., 2001).

## 2.8. Data analysis

Microbial data were log transformed using the  $\log(x + 1)$  function and calibration curves for both physicochemical and enzyme data were generated using Excel 2010 (Microsoft, USA). SPSS v.16 and SigmaPlot 11 (SPSS Inc, Chicago, USA) were used to analyse and display all data respectively. Independent Samples *t*-tests were used to analyse the relationship between treatments, time point data were analysed using one-way ANOVA and means separated using the Tukey HSD post-hoc test, and Pearson's correlations were used to analyse the relationships between parameters.

## 3. Results

### 3.1. Trial management

The liquefaction of the carcasses, with the exception of some larger bones, was completed in 56 days with whole carcasses disappearing within 28 days (Fig. 1). Despite each BV containing a similar volume of liquor and carcasses, the decomposition process was observed to differ for each vessel, with bioreduction seemingly occurring faster in BV1. Nevertheless, volume reduction did not differ significantly between vessels ( $P > 0.05$ ); with a total of 567 l and 482 l of liquor removed by evaporation from BV1 and BV2, respectively. Foaming was a problem in BV1 where, due to the poor quality lids on the bioreduction vessels, foam escaped and encased the outside of the vessel between day 7 and 15. Although foam was also produced in BV2, it was not produced to the same extent and was contained. Foam was also found in the vent from each vessel leading to the biofilter; however, the foam did not clog the biofilter or prevent it from working.

**Table 2:** DNA extraction method for bioreduction microbial communities; based on Anderson and McKay (1983). All centrifugations were done at 4 °C in an Eppendorf Centrifuge S810R (Eppendorf UK Limited, UK).

Step	Volume
Centrifuge 1 ml defrosted sample	13,000 g, 10 min
Add STE-buffer (0.1 M NaCl, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0)	700 µl
Add lysozyme (10 mg ml <sup>-1</sup> in 25 mM Tris, pH 8)	18.4 µl
Vortex for 2 s and incubate	37 °C, 30 min
Add Tris-EDTA (10mM Tris-HCl, and 1 mM EDTA, pH 8.0)	73.5 µl
Add sodium dodecyl sulphate (SDS) (20% [wt/vol] in 50 mM Tris-20 mM EDTA, pH 8.0)	44 µl
Vortex for 2 s and invert reaction tubes 6-8 times	
Incubate	50 °C, 60 min
Centrifuge	10,000 g, 10 min
Transfer supernatant into a new 1.5 ml reaction tube	420 µl
Add 5 M NaCl	42 µl
Add phenol-chloroform-isoamylalcohol (25:24:1)	420 µl
Invert reaction tubes 15-20 times	
Centrifuge	10,000 g, 10 min
Transfer upper aqueous phase into new reaction tube	200-350 µl
Add chloroform-isoamylalcohol (24:1)	1 volume
Centrifuge	10,000 g, 10 min
Transfer upper aqueous layer into new reaction tube	100-200 µl
Add isopropanol	1 volume
Incubate	4 °C, 12 h
Centrifuge	20,000 g, 20 min
Carefully remove the supernatant and keep the pellet	
Open reaction tubes, cover with a sheet of paper and air-dry in a fume hood	1 h
Remove remaining excess isopropanol using a DNA concentrator (Savant DNA120 Speedvac, Thermo Scientific, UK)	20 min
Resuspend pellets in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0)	30 µl





**Figure 1:** The sequential degradation of pig carcasses over a 56 day bioreduction trial. T = time after carcass addition in days.

### 3.2. Physicochemical analysis

A summary of the physicochemical parameters can be found in Tables 3 and 4. Half of the physicochemical parameters assayed did not significantly differ between vessels, the exceptions being pH, EC, protein, ammonium, nitrate, phosphate, total organic carbon (TOC) and total nitrogen (TN). For all exceptions, concentrations were significantly higher in BV1

than BV2 ( $P < 0.05$ ). In contrast, despite being constantly aerated, the DO concentrations were consistently below the detection limit of the meter, i.e.  $< 0.10 \text{ mg l}^{-1}$  from day 21 for BV1 and day 15 for BV2. The pH of the vessels showed two distinct phases; an initial weakly-basic phase (8.72-8.44 for BV1; 8.31-8.06 for BV2) and a subsequent moderately-basic phase from day 28 (9.09-8.89 for BV1; 8.95-9.04 for BV2). EC gradually increased until day 21 ( $4.1\text{-}10.7 \text{ mS cm}^{-1}$  for BV1;  $2.1\text{-}9.0 \text{ mS cm}^{-1}$  for BV2). However, despite a subsequent decrease after this time, at no point did EC return to starting levels (Table 3). Although, significantly correlated with EC ( $R = 0.651$ ,  $P < 0.01$  for BV1;  $R = 0.749$ ,  $P < 0.01$  for BV2), turbidity showed a slightly different trend with levels significantly higher on day 7 ( $1574 \pm 150 \text{ NTU}$  for BV1;  $1222 \pm 33 \text{ NTU}$  for BV2) and remaining fairly steady until the end of the trial (Table 3).

The moisture content within the vessels did not fall below 99% at any point during the trial. On the other hand, the organic matter content of the dry weight portion started low ( $\sim 63\text{-}66\%$ ) and progressively increased until the end of the trial ( $\sim 92\text{-}98\%$ ). Nutrient concentrations within the liquor were high throughout, with  $\text{NH}_4^+$  constituting the major nutrient (mean  $\pm$  SEM:  $1325 \pm 165$  and  $936 \pm 161$  for BV1 and BV2 respectively) followed by  $\text{PO}_4^{3-}$  and then  $\text{NO}_3^-$  (Table 4). Both vessels showed a positive correlation between  $\text{NH}_4^+$  concentration and EC ( $R = 0.719$ ,  $P < 0.01$  for BV1;  $R = 0.920$ ,  $P < 0.01$  for BV2). EC in BV1 showed an additional positive correlation with  $\text{NO}_3^-$  ( $R = 0.705$ ,  $P < 0.05$ ), whereas BV2 showed no correlation for  $\text{NO}_3^-$  but did for  $\text{PO}_4^{3-}$  ( $R = 0.738$ ,  $P < 0.01$ ). The ratio of TOC to TN was generally low, with the highest ratios obtained on day 15 (mean  $\pm$  SEM:  $2.63 \pm 0.05$  and  $2.53 \pm 0.05$  for BV1 and BV2, respectively).

### 3.3. Enzyme analysis

Enzyme activity was different for all enzymes assayed. Of the hydrolases determined fluorometrically, the acetyl esterases were the most active ( $P < 0.05$ ) (Fig. 2). In BV1, the acetyl esterase with MUF-acetate as the substrate was significantly more active than that using MUF-butyrate ( $P < 0.05$ ). This however, was not the case for BV2 ( $P > 0.05$ ) despite the highest acetyl esterase activity recorded from both vessels occurring in BV2 on day 7 using MUF-butyrate as the substrate. Indeed, both acetyl esterases showed significantly higher activity than any of the other enzymes assayed in both vessels ( $P < 0.05$ ) with the remaining enzymes showing similar levels of activity ( $P > 0.05$ ) (Figs. 2 and 3). The activity of three of

the hydrolases was assessed against corresponding substrates; phosphatase with  $\text{PO}_4^{3-}$  (Fig. 3a),  $\beta$ -D-glucosidase with carbohydrate content (Fig. 3b) and protease with protein content (Fig. 3c). Only  $\beta$ -D-glucosidase in BV1 correlated significantly with the corresponding substrate, carbohydrate ( $R = .790$ ,  $P < 0.01$ ).

**Table 3:** Descriptive physicochemical parameters of the liquor taken from the bioreduction vessels. Values represent the mean of three pseudo-replicates  $\pm$  the standard error of the mean and those followed by the same letter are considered different at the  $P < 0.05$  level according to the Tukey post-hoc test for each vessel.

	Time (days)	Temperature (°C)	pH	EC (mS cm <sup>-1</sup> )	Turbidity (NTU)	Dissolved oxygen (mg l <sup>-1</sup> )
BV1	0	26.7 $\pm$ 1.8	8.72 $\pm$ 0.01 <sup>b</sup>	4.1 $\pm$ 0.3 <sup>a</sup>	1094 $\pm$ 79 <sup>a</sup>	-
	7	40.1 $\pm$ 0.3	8.71 $\pm$ 0.04 <sup>b</sup>	9.9 $\pm$ 0.3 <sup>c</sup>	1574 $\pm$ 150 <sup>b</sup>	0.33 $\pm$ 0.10
	15	41.0 $\pm$ 0.0	8.47 $\pm$ 0.01 <sup>a</sup>	10.1 $\pm$ 0.2 <sup>c</sup>	1531 $\pm$ 84 <sup>ab</sup>	0.10 $\pm$ 0.10
	21	40 <sup>*</sup>	8.44 $\pm$ 0.02 <sup>a</sup>	10.7 $\pm$ 0.1 <sup>c</sup>	1398 $\pm$ 40 <sup>ab</sup>	< 0.10 <sup>**</sup>
	28	40 <sup>*</sup>	9.09 $\pm$ 0.02 <sup>d</sup>	10.0 $\pm$ 0.3 <sup>c</sup>	1596 $\pm$ 152 <sup>b</sup>	< 0.10 <sup>**</sup>
	42	39.7 $\pm$ 0.3	9.03 $\pm$ 0.02 <sup>d</sup>	8.2 $\pm$ 0.3 <sup>b</sup>	1353 $\pm$ 48 <sup>ab</sup>	< 0.10 <sup>**</sup>
	56	39.0 $\pm$ 0.6	8.89 $\pm$ 0.03 <sup>c</sup>	7.3 $\pm$ 0.0 <sup>b</sup>	1425 $\pm$ 55 <sup>ab</sup>	< 0.10 <sup>**</sup>
<i>Average</i>		38.1 $\pm$ 1.9	8.76 $\pm$ 0.10	8.6 $\pm$ 0.9	1425 $\pm$ 65	< 0.10 <sup>^</sup>
BV2	0	23.3 $\pm$ 0.7	8.31 $\pm$ 0.02 <sup>b</sup>	2.1 $\pm$ 0.0 <sup>a</sup>	835 $\pm$ 240 <sup>a</sup>	-
	7	37.7 $\pm$ 0.9	8.25 $\pm$ 0.01 <sup>b</sup>	5.6 $\pm$ 0.1 <sup>b</sup>	1222 $\pm$ 33 <sup>ab</sup>	0.13 $\pm$ 0.03
	15	40.7 $\pm$ 0.3	8.06 $\pm$ 0.01 <sup>a</sup>	8.5 $\pm$ 0.2 <sup>d</sup>	1707 $\pm$ 41 <sup>b</sup>	< 0.10 <sup>**</sup>
	21	43 <sup>*</sup>	8.11 $\pm$ 0.03 <sup>a</sup>	9.0 $\pm$ 0.0 <sup>d</sup>	1344 $\pm$ 89 <sup>ab</sup>	< 0.10 <sup>**</sup>
	28	40 <sup>*</sup>	8.95 $\pm$ 0.00 <sup>c</sup>	8.5 $\pm$ 0.1 <sup>d</sup>	1752 $\pm$ 153 <sup>b</sup>	< 0.10 <sup>**</sup>
	42	38.7 $\pm$ 0.3	9.03 $\pm$ 0.01 <sup>d</sup>	6.5 $\pm$ 0.0 <sup>c</sup>	1511 $\pm$ 53 <sup>b</sup>	< 0.10 <sup>**</sup>
	56	38.0 $\pm$ 0.0	9.04 $\pm$ 0.01 <sup>d</sup>	5.6 $\pm$ 0.2 <sup>b</sup>	1328 $\pm$ 23 <sup>ab</sup>	< 0.10 <sup>**</sup>
<i>Average</i>		37.3 $\pm$ 2.4	8.54 $\pm$ 0.17	6.5 $\pm$ 0.9	1386 $\pm$ 118	< 0.10 <sup>^</sup>

\* = only 1 reading taken per vessel

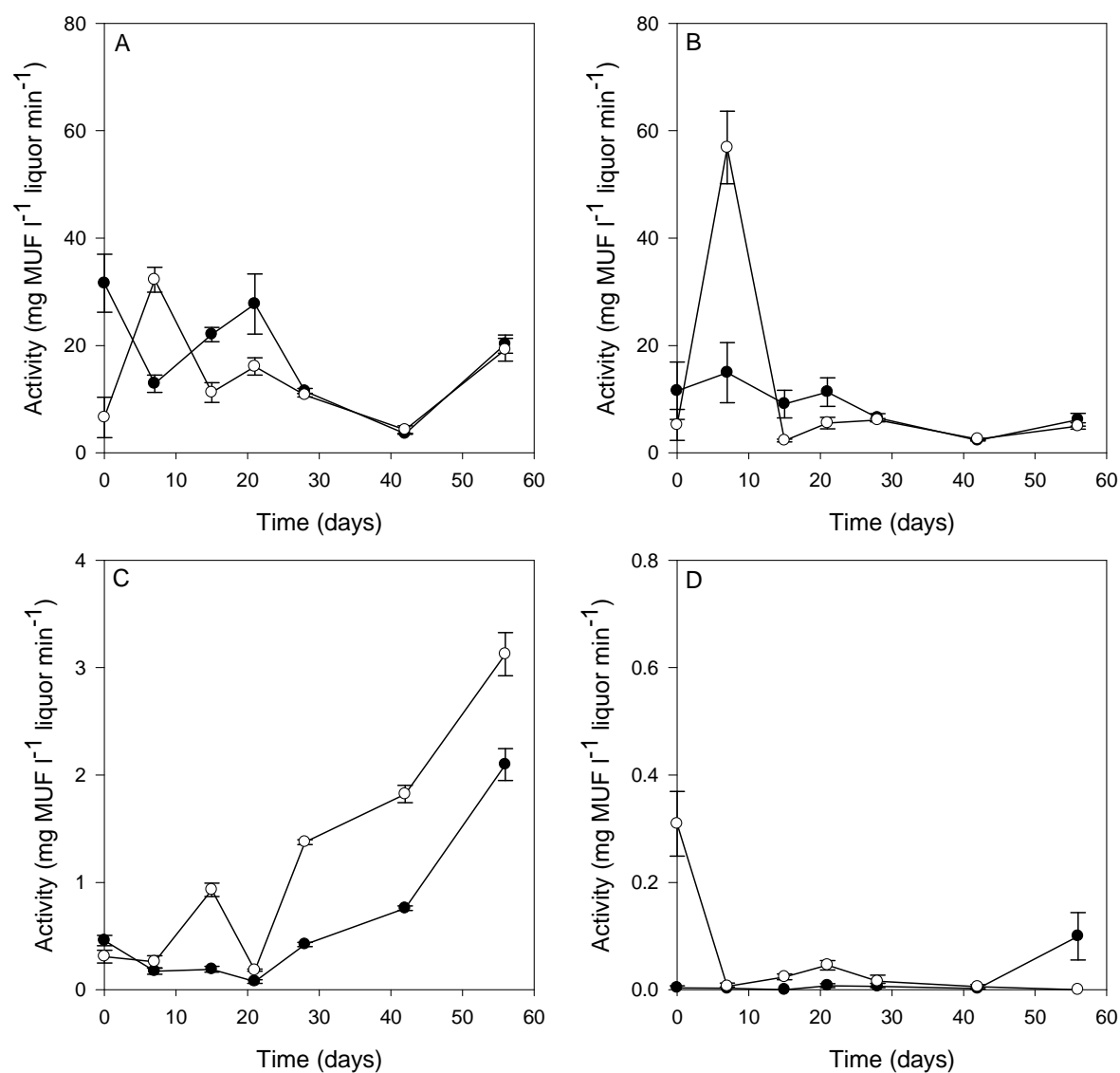
\*\* = Readings below the lower range of the instrument

^ = In this case the mode is used rather than the mean

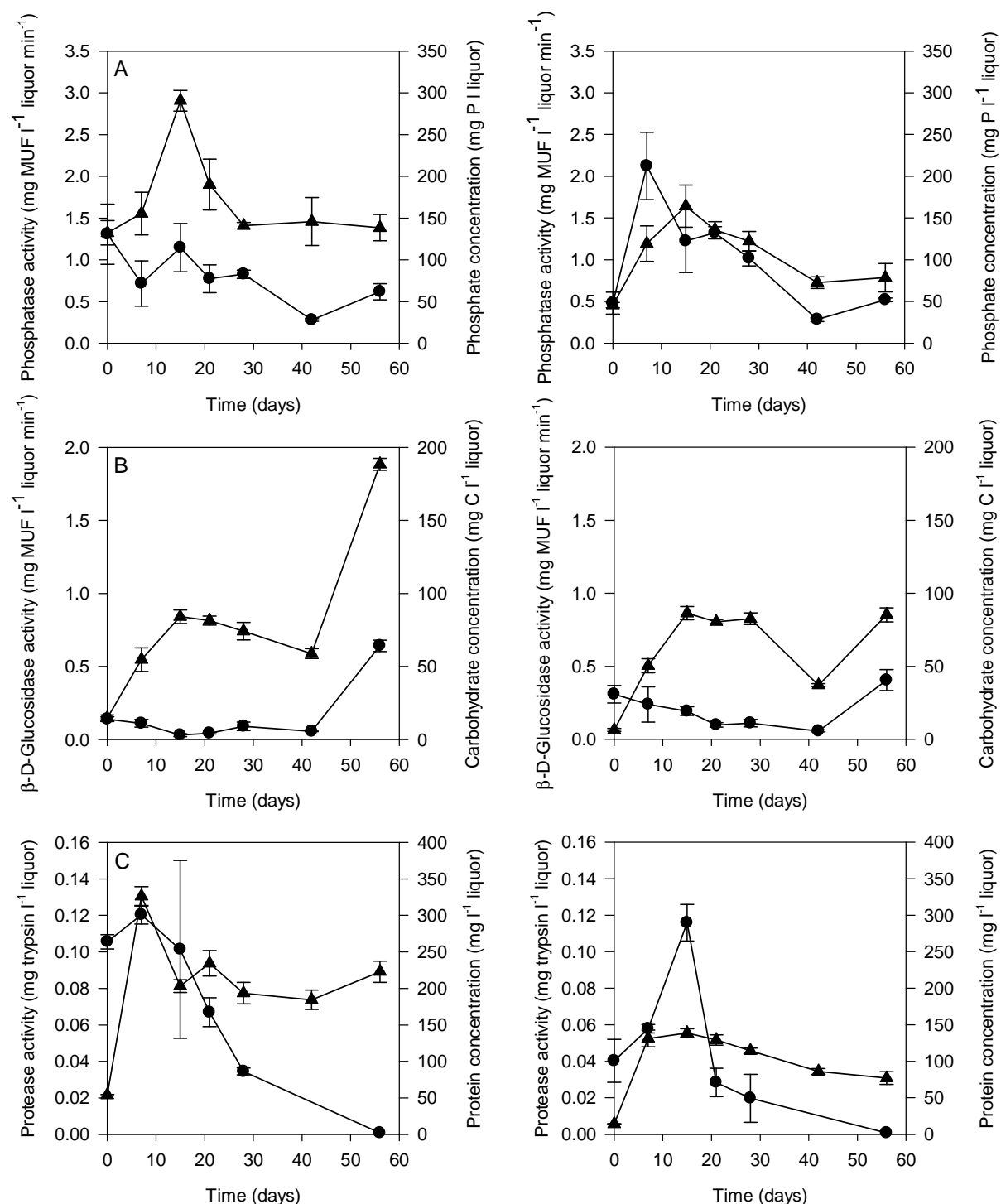
- = sample not taken

**Table 4:** The nutrient status of the liquor in the bioreduction vessels. Values represent the mean of three pseudo-replicates  $\pm$  the standard error of the mean and those followed by the same letter are considered different at the  $P < 0.05$  level according to the Tukey post-hoc test for each vessel.

	Time (days)	Moisture content (%)	Organic matter content of the dry weight portion (%)	Total organic C (mg l <sup>-1</sup> )	Total N (mg l <sup>-1</sup> )	C:N ratio	NH <sub>4</sub> <sup>+</sup> (mg N l <sup>-1</sup> )	NO <sub>3</sub> <sup>-</sup> (mg N l <sup>-1</sup> )	PO <sub>4</sub> <sup>3-</sup> (mg P l <sup>-1</sup> )
BV1	0	99.9 $\pm$ 0.0 <sup>c</sup>	62.8 $\pm$ 3.4 <sup>a</sup>	570 $\pm$ 36 <sup>a</sup>	803 $\pm$ 99 <sup>a</sup>	0.74 $\pm$ 0.13 <sup>a</sup>	433 $\pm$ 77 <sup>a</sup>	10.0 $\pm$ 1.9 <sup>a</sup>	132 $\pm$ 15 <sup>a</sup>
	7	99.6 $\pm$ 0.0 <sup>ab</sup>	74.8 $\pm$ 3.0 <sup>ab</sup>	3454 $\pm$ 29 <sup>b</sup>	3456 $\pm$ 53 <sup>f</sup>	1.00 $\pm$ 0.02 <sup>a</sup>	1333 $\pm$ 149 <sup>b</sup>	52.5 $\pm$ 3.2 <sup>cd</sup>	156 $\pm$ 26 <sup>a</sup>
	15	99.7 $\pm$ 0.0 <sup>ab</sup>	72.5 $\pm$ 1.5 <sup>ab</sup>	3582 $\pm$ 115 <sup>b</sup>	1360 $\pm$ 33 <sup>b</sup>	2.63 $\pm$ 0.05 <sup>c</sup>	1775 $\pm$ 262 <sup>b</sup>	58.5 $\pm$ 5.7 <sup>d</sup>	291 $\pm$ 12 <sup>b</sup>
	21	99.7 $\pm$ 0.0 <sup>ab</sup>	75.8 $\pm$ 0.7 <sup>ab</sup>	5330 $\pm$ 115 <sup>d</sup>	2367 $\pm$ 54 <sup>e</sup>	2.25 $\pm$ 0.08 <sup>b</sup>	1469 $\pm$ 318 <sup>b</sup>	38.4 $\pm$ 5.3 <sup>bc</sup>	190 $\pm$ 30 <sup>ab</sup>
	28	99.6 $\pm$ 0.0 <sup>a</sup>	78.3 $\pm$ 4.0 <sup>bc</sup>	3764 $\pm$ 126 <sup>b</sup>	1774 $\pm$ 5 <sup>cd</sup>	2.12 $\pm$ 0.08 <sup>b</sup>	1550 $\pm$ 40 <sup>b</sup>	25.9 $\pm$ 3.9 <sup>ab</sup>	141 $\pm$ 4 <sup>a</sup>
	42	99.8 $\pm$ 0.1 <sup>bc</sup>	84.9 $\pm$ 2.5 <sup>c</sup>	4480 $\pm$ 189 <sup>c</sup>	2108 $\pm$ 143 <sup>de</sup>	2.13 $\pm$ 0.06 <sup>b</sup>	1542 $\pm$ 57 <sup>b</sup>	17.9 $\pm$ 0.2 <sup>a</sup>	146 $\pm$ 29 <sup>a</sup>
	56	99.6 $\pm$ 0.0 <sup>abc</sup>	91.9 $\pm$ 4.6 <sup>c</sup>	3253 $\pm$ 95 <sup>b</sup>	1562 $\pm$ 105 <sup>bc</sup>	2.09 $\pm$ 0.08 <sup>b</sup>	1178 $\pm$ 118 <sup>ab</sup>	16.4 $\pm$ 2.6 <sup>a</sup>	139 $\pm$ 16 <sup>a</sup>
<i>Average</i>		99.7 $\pm$ 0.0	77.3 $\pm$ 3.5	3490 $\pm$ 557	1918 $\pm$ 320	1.85 $\pm$ 0.26	1325 $\pm$ 165	31.4 $\pm$ 7.1	171 $\pm$ 21
BV2	0	99.8 $\pm$ 0.0 <sup>a</sup>	65.9 $\pm$ 6.5 <sup>a</sup>	260 $\pm$ 4 <sup>a</sup>	328 $\pm$ 4 <sup>a</sup>	0.79 $\pm$ 0.02 <sup>a</sup>	185 $\pm$ 11 <sup>a</sup>	7.2 $\pm$ 1.8 <sup>a</sup>	46 $\pm$ 3 <sup>a</sup>
	7	99.7 $\pm$ 0.0 <sup>a</sup>	85.6 $\pm$ 4.8 <sup>bc</sup>	1714 $\pm$ 9 <sup>c</sup>	1978 $\pm$ 20 <sup>e</sup>	0.87 $\pm$ 0.00 <sup>a</sup>	733 $\pm$ 103 <sup>b</sup>	5.6 $\pm$ 1.6 <sup>a</sup>	119 $\pm$ 21 <sup>abc</sup>
	15	99.7 $\pm$ 0.0 <sup>a</sup>	84.3 $\pm$ 3.6 <sup>bc</sup>	4016 $\pm$ 110 <sup>e</sup>	1588 $\pm$ 15 <sup>d</sup>	2.53 $\pm$ 0.05 <sup>e</sup>	1406 $\pm$ 159 <sup>c</sup>	18.5 $\pm$ 4.0 <sup>b</sup>	164 $\pm$ 25 <sup>c</sup>
	21	99.7 $\pm$ 0.0 <sup>a</sup>	74.8 $\pm$ 2.6 <sup>ab</sup>	4257 $\pm$ 199 <sup>e</sup>	2008 $\pm$ 83 <sup>e</sup>	2.12 $\pm$ 0.01 <sup>d</sup>	1387 $\pm$ 84 <sup>c</sup>	8.6 $\pm$ 0.8 <sup>a</sup>	136 $\pm$ 10 <sup>bc</sup>
	28	99.4 $\pm$ 0.2 <sup>a</sup>	79.4 $\pm$ 2.5 <sup>abc</sup>	2053 $\pm$ 44 <sup>cd</sup>	1275 $\pm$ 17 <sup>c</sup>	1.61 $\pm$ 0.02 <sup>c</sup>	1039 $\pm$ 53 <sup>bc</sup>	3.1 $\pm$ 0.8 <sup>a</sup>	122 $\pm$ 12 <sup>bc</sup>
	42	99.7 $\pm$ 0.0 <sup>a</sup>	94.8 $\pm$ 1.9 <sup>c</sup>	2304 $\pm$ 31 <sup>d</sup>	1338 $\pm$ 48 <sup>c</sup>	1.73 $\pm$ 0.04 <sup>c</sup>	1058 $\pm$ 59 <sup>bc</sup>	10.4 $\pm$ 0.3 <sup>ab</sup>	73 $\pm$ 7 <sup>ab</sup>
	56	99.8 $\pm$ 0.0 <sup>a</sup>	97.6 $\pm$ 1.9 <sup>c</sup>	1261 $\pm$ 41 <sup>b</sup>	1036 $\pm$ 37 <sup>b</sup>	1.22 $\pm$ 0.01 <sup>b</sup>	742 $\pm$ 26 <sup>b</sup>	9.0 $\pm$ 0.3 <sup>a</sup>	78 $\pm$ 17 <sup>ab</sup>
<i>Average</i>		99.7 $\pm$ 0.0	83.1 $\pm$ 4.2	2266 $\pm$ 544	1364 $\pm$ 220	1.55 $\pm$ 0.24	936 $\pm$ 161	8.9 $\pm$ 1.9	105 $\pm$ 16



**Figure 2:** The activity of (A) acetylcysterase (MUF-acetate), (B) acetylcysterase (MUF-butyrate), (C) lipase and (D) sulphatase during the bioreduction of pig carcasses in bioreduction vessels 1 (●) and 2 (○). Values have been corrected for background fluorescence and represent the mean of three pseudo-replicates  $\pm$  standard error of the mean.



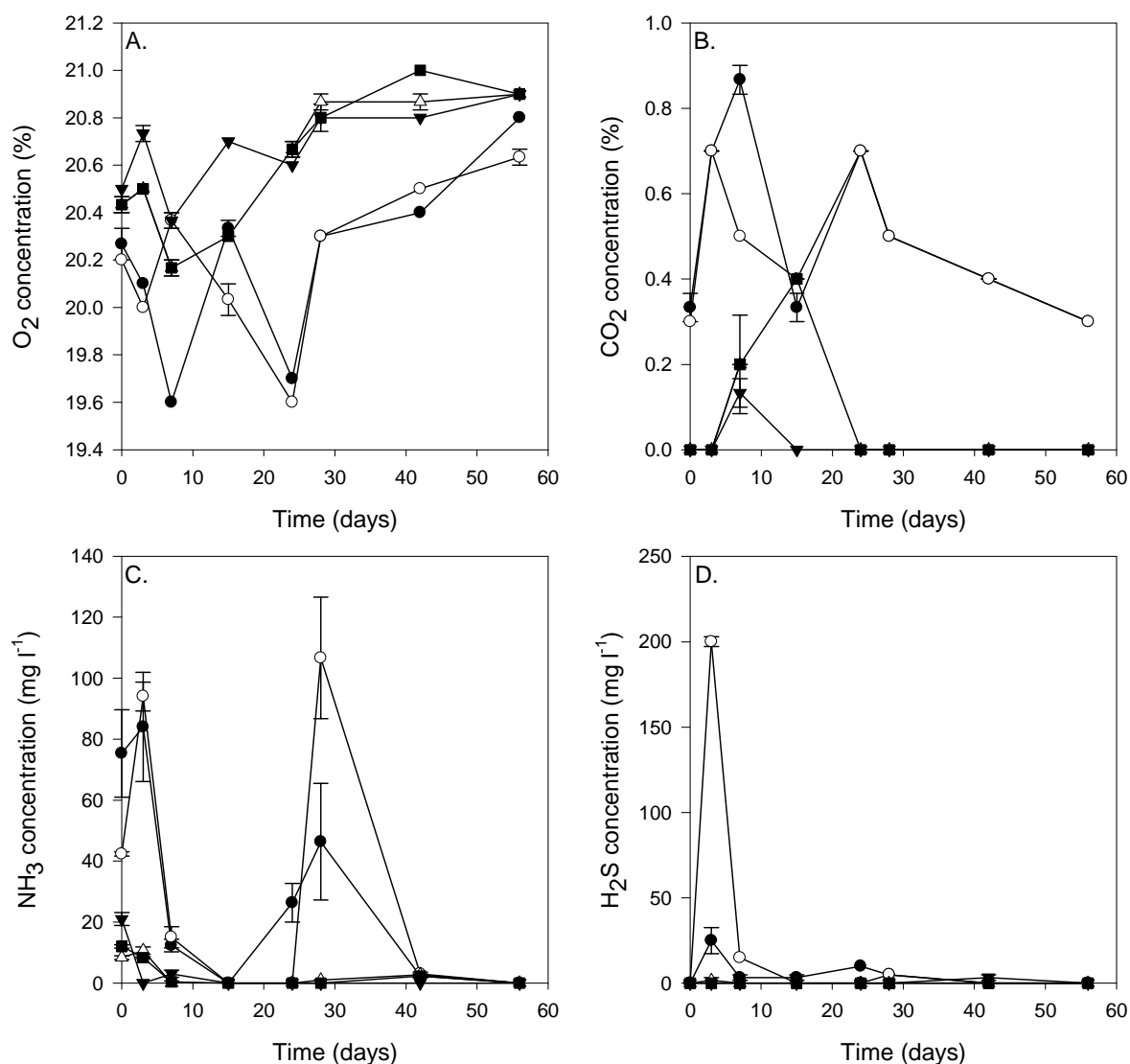
**Figure 3:** The activity of three enzymes (●) and the concentration of the assumed substrates (▲) in bioreduction vessels 1 and 2: (A) phosphatase and its relationship with phosphate concentration; (B) β-D-glucosidase and its relationship with carbohydrate concentration; and (C) protease activity and its relationship with protein concentration. Values have been corrected for background fluorescence and represent the mean of three pseudo-replicates ± standard error of the mean

### 3.4. Gas emissions

Gaseous emissions were measured from the outlet pipes of the BVs, at the surface of the biofilter, 10 m downwind and 10 m upwind of the BVs. O<sub>2</sub> levels in the BVs remained fairly high throughout, with a minimum recorded concentration of 19.6% (Fig. 4a) and at no point were O<sub>2</sub> levels from the BVs statistically different to any of the other locations sampled ( $P > 0.05$ ). Nevertheless, gases associated with anaerobic conditions (CO<sub>2</sub>, NH<sub>3</sub> and H<sub>2</sub>S) were recorded at various points throughout the trial (Fig. 4b-4d). Despite this, no CH<sub>4</sub> was detected at any point (data not shown; detection limit <0.1%). Both CO<sub>2</sub> and NH<sub>3</sub> showed two peaks in emissions for the BVs, the first on days 3-7 and the second on days 24-28 (Fig 4b and 4c); resulting in mean CO<sub>2</sub> emissions statistically higher than those of other locations ( $P < 0.05$ ). Similarly, BV1 had statistically higher mean NH<sub>3</sub> emissions to all locations ( $P < 0.05$ ) with the exception of BV2 ( $P > 0.05$ ). The highest H<sub>2</sub>S emissions were recorded on day 3 (25 mg l<sup>-1</sup> for BV1 and 200 mg l<sup>-1</sup> for BV2). However, mean H<sub>2</sub>S emissions were not statistically different between the vessels ( $P > 0.05$ ). Nevertheless, H<sub>2</sub>S emissions from BV2 were higher than all other locations ( $P < 0.05$ ).

### 3.5. Odour

Odour was detected from the study site at all time points before the vessels were opened. All odour results were obtained by one person (Ceri Gwyther) and are meant as a guide rather than an absolute value. The highest odour was detected on day 3 whilst the bioreduction vessels were shut. In this instance, the odour was classified as 5 according to the scale of Jiang et al. (2006) (“The odour is annoying. Exposure to this level would be considered undesirable”). It was envisaged that were the vessels opened, the odour within the vessels would have reached at least the next highest level on the scale. A second peak in odour occurred on day 15. In this instance, odour within the vessel was recorded as a 5 and a 4 in the immediate vicinity whilst the loading hatches were closed (“The odour character is clearly recognizable. Long time exposure to the odour is not tolerable”).

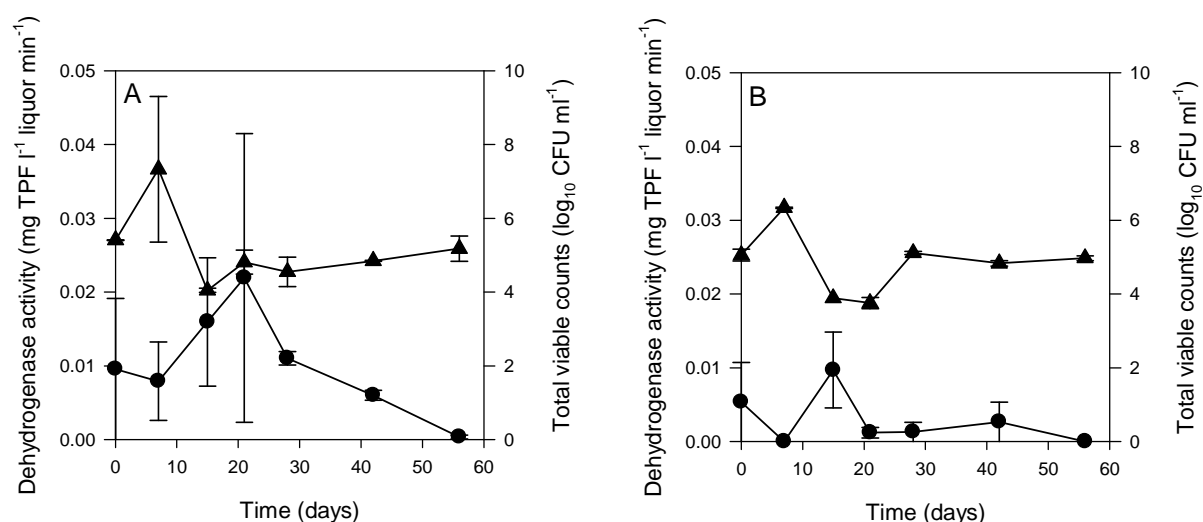


**Figure 4:** The emissions of (A) O<sub>2</sub>, (B) CO<sub>2</sub>, (C) NH<sub>3</sub>, and (D) H<sub>2</sub>S from the bioreduction vessels (BV) 1 (●) and 2 (○), biofilter (▼) and 10 m upwind (Δ) and downwind (■) of the vessels. Values represent the average of three replicates ± the standard error of the mean.



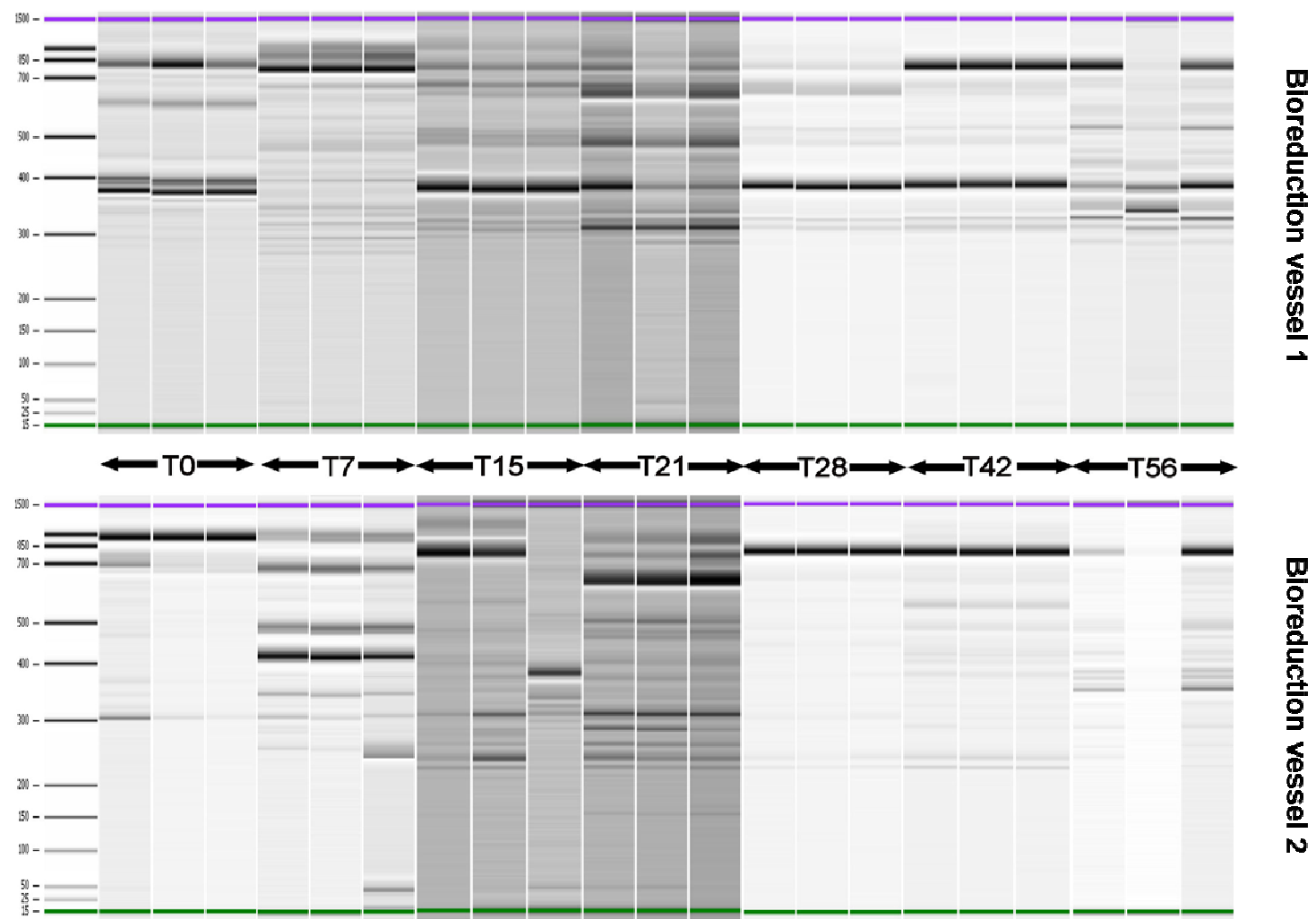
### 3.6. Microbial analysis

TVC numbers peaked on day 7 for both vessels, reaching a maximum count of 7.33  $\log_{10}$  CFU  $\text{ml}^{-1}$  in BV1 and 6.34  $\log_{10}$  CFU  $\text{ml}^{-1}$  in BV2 (Fig. 5). However, for BV1, these values were not statistically significant when compared to other time points ( $P > 0.05$ ). In contrast, this peak was significant for BV2 ( $P < 0.05$ ) with levels proceeding to fall below starting counts on days 15-21 ( $P < 0.05$ ) before returning back to starting levels ( $P < 0.05$ ) and remaining constant from day 28 onwards ( $P > 0.05$ ). Despite this difference in pattern, mean numbers were not statistically different between vessels ( $P > 0.05$ ). DA on the other hand, peaked on day 15 for BV2 and day 21 for BV1 (0.01 and 0.02  $\text{mg tyrosine l}^{-1} \text{min}^{-1}$  respectively). No correlation was observed between TVC and DA ( $R = 0.013$ ,  $P > 0.05$  for BV1;  $R = -0.240$ ,  $P > 0.05$  for BV2) (Fig. 5).

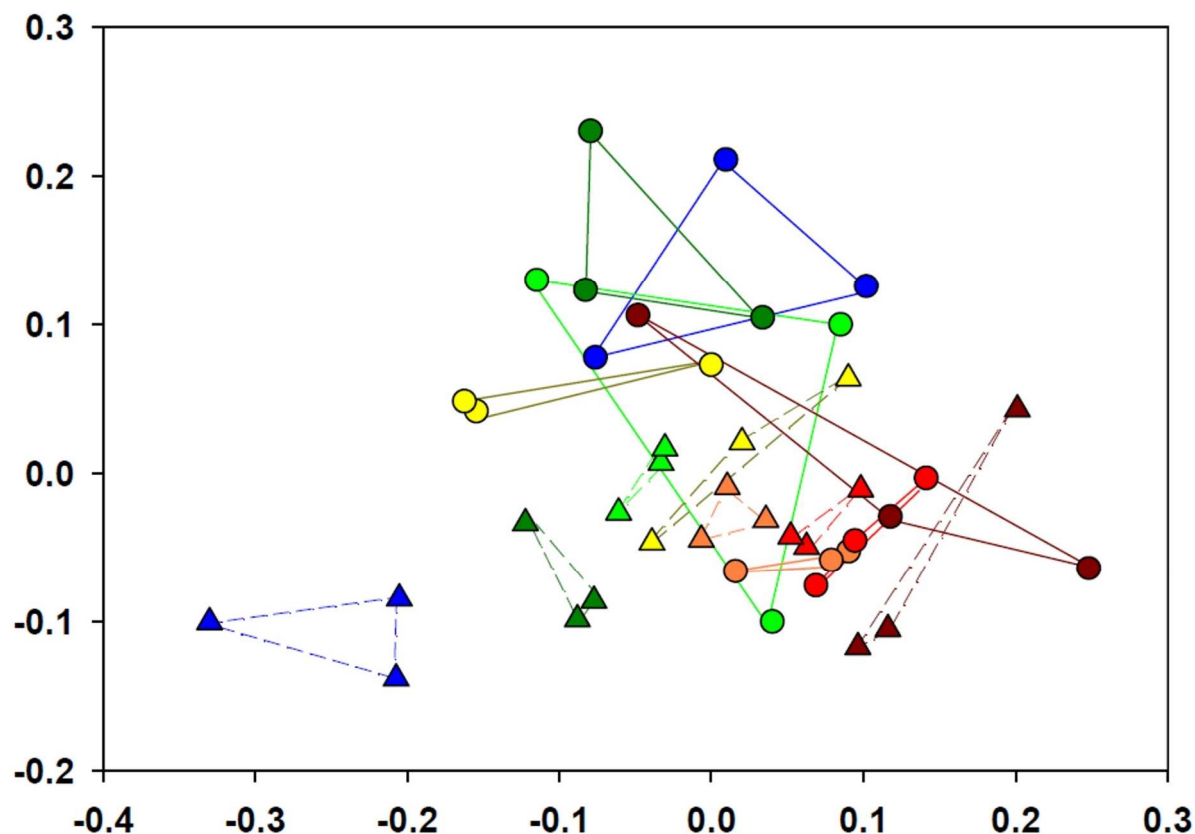


**Figure 5:** The activity of dehydrogenase (●) and its relationship with total viable counts (▲) in bioreduction vessel 1 (A) and 2 (B). Values have been corrected for background activity and represent the mean of three pseudo-replicates  $\pm$  standard error of the mean.

The ARISA fingerprints for each vessel can be found in Fig. 6. In this trial, both vessels started with different microbial inoculum (Fig. 7). Microbial communities in both vessels quickly started changing within the first 21 days. In both cases, communities converged and formed very similar climax communities on days 28 to 42. After 42 days of incubation, microbial communities changed again until the end of the trial on day 56 (Fig. 7). ARISA profiles for the final stages showed an increase in band numbers (Fig. 6) and developing divergence between both vessels.



**Figure 6:** The change in microbial communities over time (T = time in days) in two bioreduction vessels containing pig carcasses.



**Figure 7:** Non-metric multidimensional scaling analysis of the microbial communities in the two bio-reduction vessels (BV), where triangles represent BV1 and circles represent BV2. The different colours denote the following sampling times in days: blue = 0; dark green = 7; green = 14; yellow = 21; orange = 28, red = 42 and dark red = 56.

#### 4. Discussion

The degradation of whole pig carcasses within 28 days is consistent with studies using sheep carcasses (Gwyther et al., 2012; Williams et al., 2009). However, it is likely that the decomposition of swine carcasses was even more accelerated in this trial, but the production of excessive amounts of foam during the first 15 days prohibited the identification of carcass remains (Fig. 1). Given that the degradation rate of pig carcasses was similar to that of sheep, and the abundant availability of space within the vessels, it is estimated that at least 2,816 kg of pig carcasses can be stored using this method per annum, based on extrapolation of the figures provided by Williams et al. (2009).

Foaming was a problem during the period of day 7 to day 15 and therefore brings into question the biosecurity of the technology for the storage of pig carcasses. However, foaming is also a known problem in existing waste treatment processes, both aerobic and anaerobic (Cumby, 1987; Ganidi et al., 2009; Mohaibes and Heinonen-Tanski, 2004). Ganidi et al. (2011) showed that shock loading during anaerobic digestion (AD) of wastewater sludge caused foaming, with loading rates of  $\geq 2.5 \text{ kg VS m}^{-3}$  initiating foam formation. Similar shock loading has been observed in the aeration of animal slurries (Cumby, 1987). It is highly likely therefore, that the high number of carcasses added to each BV in a single instance caused similar shock loading. No foaming was observed in a previous trial using sheep carcasses undertaken by these authors (Gwyther et al., 2012) despite a higher initial loading rate of up to 206 kg per vessel. However, it was an issue reported by Williams et al. (2008), in a similar trial also using sheep carcasses. Consequently, optimum loading rates need to be determined for all species of carcasses that could potentially be bio-reduced; e.g. sheep, pigs and poultry. In addition to reducing loading rates, foam can be managed using a range of techniques such as using rotating blades to cut and break the bubbles in the foam; siphoning off the foam and disposing of it separately; the addition of antifoam reagents; the use of oxygen rather than air to aerate the vessels and by creating more capacity within the vessels to contain the foam (Mohaibes and Heinonen-Tanski, 2004; Cumby, 1987). Foaming therefore, is a transitory problem that can be overcome in future iterations of the bio-reduction vessel design.

The liquor produced was nutrient-rich and moderately alkaline, similar in composition to the liquor produced from sheep bio-reduction (Williams et al., 2009). As in the study by Williams et al. (2009), ammonification was presumed to be the major process in the nitrogen cycle with low DO concentrations and low nitrifier populations preventing the complete nitrification of  $\text{NH}_4^+$ . However, the  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations were considerably higher in the bio-reduction of pig carcasses compared to the bio-reduction of sheep carcasses (Williams et al., 2009) indicating that liquor from different species will have different physicochemical properties. In addition, considering the statistical differences in nutrient content between vessels (section 3.2), the starting inoculum and carcasses added will play a prominent role in nutrient dynamics, altering the physicochemical properties from vessel to vessel regardless of the animal species contained therein.

The low DO conditions in the liquor may have also been the key parameter responsible for lipid decomposition. During the breakdown of carcasses, neutral fat is hydrolysed into

fatty acids which may then be oxidised or hydrolysed further depending on the oxygen status of the environment. Under sufficiently aerobic conditions, fatty acids will be further oxidised to aldehydes and ketones (Forbes, 2008). As both vessels showed high levels of acetylsterase activity during the first 21 days with a concurrent reduction in pH (Table 3), it is hypothesised that in the bioreduction of pig carcasses, hydrolysis of the lipids was the predominant degradation pathway and the release of fatty acids contributed to the lowered pH of the bioreduction liquor. When acid is added to a nutrient-rich substrate such as slurry, or in this case bioreduction liquor, volatilisation of CO<sub>2</sub> can occur resulting in the production of foam and neutralising the base buffering capacity of HCO<sub>3</sub><sup>-</sup> (Vandré and Clemens, 1997). This is one explanation of both the appearance of foam as described above, but also the peak of CO<sub>2</sub> emission observed on day 7 for BV1 and day 3 for BV2 (Fig. 4B).

Other gases associated with anaerobic conditions were also recorded at various times throughout the trial (Fig. 4); most notably, H<sub>2</sub>S and NH<sub>3</sub> emissions. It is possible that alongside the volatilisation of CO<sub>2</sub> described above, the carcasses themselves retained anaerobic niches until decomposition was sufficiently advanced to allow the penetration of oxygen deeper into the remaining carcass material, thus generating H<sub>2</sub>S. CO<sub>2</sub> showed a second peak in both vessels occurring at day 24. On both occasions, CO<sub>2</sub> emissions were followed by an increase in NH<sub>3</sub> emissions (Fig. 4B). It is hypothesised that the initial peak in NH<sub>3</sub> was due to the accumulation of NH<sub>4</sub><sup>+</sup> during an ammonification and concurrent nitrification phase (Table 4). As the pH of the vessels was moderately basic and the temperatures were consistently at 40 °C (Table 3), the NH<sub>4</sub><sup>+</sup> was volatilised as NH<sub>3</sub> gas. However, the high microbial activity observed during days 15-24 (Fig. 5) potentially caused a shift in the oxygen status of the liquor from aerobic to anaerobic, as what limited oxygen there was available in the liquor was removed during the metabolism of these nutrients (Fig. 4A and Table 4). Thus after this point, approximately day 24, denitrification started to occur. However, denitrification did not proceed past the point of using NO<sub>2</sub><sup>-</sup> as the final electron acceptor in the electron transfer chain, which caused a second release of NH<sub>3</sub> gas (Konhauser, 2007), again due to the high pH and temperature. As the NO<sub>3</sub><sup>-</sup> started to become depleted (Table 4) and the oxygen levels once more increased (Fig. 4A), this switch was reversed and NH<sub>3</sub> gas no longer produced.

The high concentration of nutrients found in the liquor is one possible explanation for the low enzyme activity observed, as a high concentration of catabolites has been found to inhibit enzyme activity (Morgenroth et al., 2002). Other explanations for low enzyme activity

include: different enzymes acting on the same substrate; the methodologies used in the enzyme assays and the constantly changing nature physicochemical parameters effecting substrate saturation (Burns, 1982; Gianfreda & Ruggiero, 2006); and the redox constrained nature of the liquor. As well as increasing available space for new carcasses in the vessels by improving bioreduction efficiency, increasing enzyme activity may theoretically prevent the build-up of intermediate products which may be one cause of foam production (Ganidi et al., 2011) which may in turn reduce the emission of volatile organic compounds (VOCs) and other anaerobic gases which are associated with bad odours (Akdeniz et al., 2010). Improved enzyme efficiency in future starting inoculums may be achieved with the addition of enzyme preparations or by altering the microbial community within the vessel. Nevertheless, the data from this study showed that despite both vessels having differing starting communities, a similar climax community was achieved in both vessels (Fig. 7) coinciding with the breakdown of the soft tissue and the abundance of nutrients. This suggests that it may be difficult to manipulate the community without sufficiently changing the physicochemical parameters within the vessel. However, these are certainly paths which should be investigated in order to further enhance the efficiency of bioreduction.

## **5. Conclusions**

Despite the continued aeration of the bioreduction vessels, the liquor was redox-constrained producing malodours, a build up of  $\text{NH}_4^+$  and as a consequence, a reduction in enzyme efficiency. A by-product of these processes was a build-up of foam in the first few weeks of operation. Nevertheless, it may be possible to increase enzyme activity by either increasing the aeration efficiency or by supporting the microbial community using artificial inoculations and adding enzyme preparations. The resulting improvements in enzyme activity may reduce the build-up of intermediate products and thus prevent foaming as well as speeding up the process of bioreduction.

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## 5. General discussion of results

### 5.1. Biosecurity

Whilst at first glance, traditional culturing techniques for enumerating pathogens appears to be cost-effective and practically straightforward, in hindsight there were many inherent methodological problems associated with this approach. For example, all selective media used showed a high level of contaminating colonies which made enumeration of the target organism difficult. *Salmonella* spp. are notoriously difficult to enumerate (Malorny et al., 2008) and confirmation is labour-intensive, time-consuming and can also lead to erroneous results (Bennett et al., 1998; Malorny et al., 2008). Different media were used in the pathogen trials to try and overcome the problem of background contamination. Xylose-lysine-desoxycholate agar (XLD) was originally used in the laboratory trial with Brilliant Green Agar (BGA) used for bioaerosol analysis. In the first pathogens field trial (under standard operating conditions), both XLD and BGA were used in *Salmonella* spp. enrichment according to ISO 6579:2002, whereas in the second field trial (under simulated breakdown conditions) immunomagnetic separation (IMS) was used to improve *Salmonella* spp. selectivity before plating onto XLD containing the antibiotic novobiocin, which inhibits gram-positive bacterial growth. Despite this, not even the use of IMS removed the contamination completely although it was improved, though whether this improvement was due to the IMS or the addition of novobiocin was not clear. *Salmonella* spp. was not the only problematic pathogen. *E. faecalis* was not able to be specifically isolated on the chosen Slanetz and Bartley media (SBM). In fact, there are no media that selectively isolate *E. faecalis* to the exclusion of other *Enterococcus* spp. known to the author. However, as a thermotolerant bacterium, *E. faecalis* is specifically required to be enumerated according to the EFSA guidelines (Bohm, 2008). Indeed, the use of culturing techniques in this case could have led to erroneous conclusions being formed if the analysis was not validated by 16S rRNA sequencing. Culturing techniques showed an increase in *Enterococcus* spp. from day 28 which was assumed to be *E. faecalis* due to the high inoculation levels. However, 16S rRNA sequencing showed that although there was an increase in *Enterococcus* spp., there was a concurrent decrease in *E. faecalis* specifically.

The use of many PCR techniques would also have provided very little information as they do not tell the researcher if the DNA is from viable or dead organisms (Quilliam et al., 2011). However, more modern techniques such as real-time reverse transcriptase PCR (real-time RT-PCR) overcome these problems by reverse transcribing target RNA into cDNA and

replicating the cDNA by the quantitative method, real-time PCR (Quilliam et al., 2011). As with any technique there are limitations, such as choosing primers that allow one copy of the target sequence to be replicated per organism and the ability to extract the DNA from the sample matrix. These methods may also be time consuming and expensive but the ability to freeze the DNA gives the researcher more freedom and scope to optimise their workload. If the biosecurity implications of other novel methods of fallen stock storage and disposal are going to be proposed in future, it is suggested that culturing techniques are avoided unless high levels of contamination can be minimised and real-time RT-PCR used instead.

Nevertheless, despite the problems associated with the microbiological analysis, bioreduction was shown to be biosecure, both with respect to pathogen survival in the liquor and from bioaerosols. Indeed, the pathogen related field-scale trials (Article 3) showed numbers of all pathogens monitored reduced sufficiently to satisfy the criteria of the more stringent requirements of the *disposal* option guidelines (Bohm, 2008) for sheep carcasses. Further, the vessels are not accessible to pests and scavengers which could spread disease agents and the area can be fenced off to restrict access to other animals and to humans. However, without the analysis of vegetative bacteria, protozoa and prions (Bohm, 2008) it is unlikely that bioreduction will be accepted as a *disposal* option. Nevertheless, the data collected shows that bioreduction of sheep carcasses more than satisfies the requirements of EFSA for on-farm *storage* prior to disposal and therefore should be favourably considered. Biosecurity issues have been raised however, due to the release of foam during the bioreduction of pig carcasses (Section 5.3.2) and further work is needed to assess the biosecurity implications of foam release before the technology is approved for the more volatile pig carcasses.

## 5.2. Environmental implications

### 5.2.1. Carbon footprint

The work performed in Article 5 showed that the use of electricity to heat a bioreduction vessel is inefficient and gives bioreduction a large carbon footprint, particularly when compared to the current methods of fallen stock disposal; i.e. the central collection service and the use of ‘Dolav’ boxes to securely store the carcasses. There are ways to improve the insulation of the vessels and thus reduce the amount of electricity required to heat them, such as making them double skinned with a central insulation layer. However,

perhaps the most effective way to heat the vessels is to treat them as a large-scale boiler and heat using oil; although this may necessitate a full-scale redesign of the vessels. Whilst it was shown that the contribution of bioreduction to the carbon footprint of the whole farm would be small (<2%), there are other negative associations, namely the high cost of electricity. This cost would inevitably be prohibitive to the uptake of bioreduction by farmers.

The carbon footprint aside, bioreduction has some positive environmental aspects. Burial of carcasses, both animal and human, has been associated with the pollution of ground- and freshwater (NABC, 2004). The use of a fibreglass vessel contains any nutrients and pathogens that could be released due to the decomposition of carcasses and therefore prevents the contamination of waterbodies. Further, the vessels are placed in a thick ‘Visqueen’ layer, on a bed of sand (Williams et al., 2008) which further limits the likelihood of puncturing and resultant leakage of liquor. This may make bioreduction a particularly suitable storage choice in remote areas where burial and burning is still acceptable under the ABPR. Particulate matter release is also thought to be minimal for two reasons; solid material generally sinks to the bottom of the vessels and any particles release will become trapped in the compost and woodchip biofilter. However, as this has yet to be ratified scientifically, more research is needed into the environmental considerations of bioreduction.

#### *5.2.2. Land-application of bioreduction liquor*

Looking at the physicochemical characteristics of the liquor from the sheep trial run by Williams et al. (2009), the laboratory-scale bioreduction trial in Article 2 (Appendix II, Table AIIa), the sheep trials run in Article 3 (Appendix II, Table AIIb) and the pig trial run detailed in Article 6, it is apparent that the nature of the liquor is different each time, depending on the characteristics of the feedstock, i.e. carcasses and inoculum. Nevertheless, in all cases,  $\text{NH}_4^+$  was the predominant inorganic nutrient produced. However, even in replicates of the same treatment, the concentration varied greatly. This high  $\text{NH}_4^+$  concentration has implications should bioreduction ever be considered as a disposal option and the liquor applied to land as even small concentrations of  $\text{NH}_4^+$  can be toxic to plants. However, it is likely that the  $\text{NH}_4^+$  will quickly oxidised into  $\text{NO}_3^-$  during nitrification, which is more readily assimilated by plants (Schlesinger, 1997). High  $\text{NH}_4^+$  concentrations are also found in pig slurry which is regularly applied to land, with concentrations ranging from 760  $\text{mg l}^{-1}$  (assuming 1  $\text{mg kg}^{-1}$  wet weight is equal to 1  $\text{mg l}^{-1}$ ) (Popovic and Jensen, 2012) to 7,570  $\text{mg l}^{-1}$  (again assuming

that 1 kg M<sup>-3</sup> is equal to 1 mg l<sup>-1</sup>) (Yagüe and Quílez, 2012). Slurry application to land poses a range of environmental problems such as the leaching of NH<sub>4</sub><sup>+</sup> to waterbodies and the accumulation of trace metals in the topsoil (Popovic and Jensen, 2012). Therefore, bioreduction liquor potentially poses similar problems, although NH<sub>4</sub><sup>+</sup> concentrations in the liquor are on the lower end of the concentration range (Article 6).

In acidic areas, lime is often applied to reduce the acidity of soils. The addition of lime to bioreduction liquor prior to land-application could potentially reduce pathogen loadings as has been shown for other wastes such as abattoir waste (Avery et al., 2009). However, given the potential for TSE transmission from sheep (Anon, 2009), this type of liquor would not be allowed to be used as a fertiliser, at least without prior treatment. To date, pigs have shown no natural infection by prions, the agent responsible for TSE and indeed, no pigs have been infected via the oral route experimentally (EC SSC, 1999). Therefore, despite still being classified as high-risk according to the European Community Scientific Steering Committee (EC SSC) (EC SSC, 1999), it is the opinion of this author that pig (and poultry) liquor should be acceptable for land-spreading. Indeed, the EC SSC state that in some circumstances Category 2 materials, which include fallen stock that does not contain specified risk material, may be transformed into organic fertilisers (EC SSC, 1999). However, it is necessary that bioreduction must firstly be accepted as a *storage* option before it can be considered as a *disposal* option.

### 5.3. Practical considerations

#### 5.3.1. Accelerants and starter inoculum

As with any novel technology, there are gaps in our understanding of the processes involved. For example, the accelerant that is recommended by previous researchers (Gutiérrez et al., 2003) has not been ratified in the scientific literature. Therefore, adding an accelerant that does not necessarily enhance the rate of degradation may be an unnecessary cost to the end user. Therefore, five commercial accelerants including the recommended accelerant, Gel-60<sup>®</sup>, liquor from a previous bioreduction and soil were assessed as potential bioreduction accelerants. Not one accelerant met all the criteria specified as increasing decomposition rates; namely, an overall increase in mass loss and moisture content and an increase in protein and amino acid concentration when compared to the control. The accelerant that met the most criteria was the recommended accelerant, Gel-60<sup>®</sup>; however, this

was at a dose rate of 500-times what was recommended by Gutiérrez et al. (2003) and therefore would be 500-times the cost. The fact that bioreduction liquor failed to promote the acceleration of decomposition was surprising as other organic waste disposal options such as composting and anaerobic digestion retain a proportion of the previous medium to ‘inoculate’ the next batch (Gerardi, 2003; Sundberg & Jonsson, 2005). This suggests that the final microbial community within bioreduction liquor is not necessarily the best suited to the decomposition of large portions of carcasses and that the intrinsic microbial community is of greater significance.

The initial microbial communities in the field-scale bioreduction of pig carcasses were different in the two vessels studied (Article 6). However, once the soft tissue became separated from the bone and high nutrient concentrations were seen, the communities converged. Subsequently, near the end of the 56 day study, the microbial communities diverged once more. There did not seem to be a single band that remained through the entire trial which would indicate an organism(s) that was important throughout the entirety of the decomposition process. However, further analysis using denaturing gradient gel electrophoresis (DGGE) and 16S rRNA sequencing of the bands could help to improve our understanding of the microbial community and potentially provide information that could lead to the production of a starter inoculum specifically for bioreduction.

### *5.3.2. Foaming*

Excessive foaming was observed in the vertically-aligned bioreduction vessels, both with sheep and pig carcasses (Article 6; Williams et al., 2008). In contrast, excessive foaming was not seen in these same vessels during the field trial using on-farm standard operating procedures (Article 3). In all cases, the vessels were heated to 40 °C and aerated at the standard rate of 50 kPa for 45 min h<sup>-1</sup>. The reason for excessive foaming is not yet understood although it is likely it is related to the aeration rate (Article 6). In a review of the literature on foaming during anaerobic digestion, Ganidi et al. (2009) found that mixing vessels using gases ultimately resulted in excessive foaming when compared to mechanical mixing. It may also relate to the total amount of carcasses in the vessel at any one time, known as ‘shock loading’ (Ganidi et al., 2011). Therefore, the optimal loading of carcasses still needs to be calculated to a) avoid excessive foaming during period of high mortality such as the lambing period and b) to ensure that shock loading does not occur after periods of no carcass additions

or during the start up of new vessels. Whilst not resolving the issue, the foam may be contained using the sturdier lid design of the horizontally-aligned bioreduction vessels. There are other existing methods of dealing with foam production such as the use of blades to cut the bubbles (Mohaibes & Heinonen-Tanski, 2004) but these are likely to not only increase the complexity of the vessels but also the cost, not to mention inhibiting the addition of carcasses to the vessels given their current design. Further research is therefore needed to identify the reasons behind foam production and methods to either prevent or mitigate it.

### *5.3.3. Mechanical mixing*

Although not used in any of the experiments in this thesis, the use of the mixing paddles in the horizontally-aligned vessels was tested. However, the paddles were too small and carcasses caught on the paddles. Indeed, a major problem with the horizontally-aligned vessels was the clumping of carcasses near the loading hatch. If this style of vessel is to be continued, the loading hatch should be positioned more centrally. The second problem was that the rotor used to turn the blades was designed for areas with little rainfall and as a result the motor rusted soon after installation. Therefore, if mechanical mixing is going to be used in future then the blades need to be re-designed and the electrical components improved to meet standards required in areas of high rainfall. However, mechanical mixing may be the answer to the foaming problem outlined above and could prevent the clogging of aeration vents reported by Williams et al. (2008). Nevertheless, in order to remain aerobic and prevent the release of malodorous gases, aeration of the liquor still needs to occur. The trade-off between aerating the vessels and the production of foam therefore still needs to be addressed before the vessels become commercially available.

### *5.3.4. Odour*

Although odour released during the early stages of pig decomposition were related to  $H_2S$  and  $NH_3$  production, subsequent odours showed no correlation (Article 6). Therefore, it is advised that analysis of the volatile organic compounds (VOCs) is conducted to determine which odorous compounds are released. The lids of the horizontally-aligned vessels were far superior at containing malodorous gases compared to those on the vertically-orientated vessels. Indeed, no complaints were made by members of staff during the pathogen trials in Article 3 when carcasses were added to the horizontally-aligned vessels; in comparison,

during Article 6 when only the vertically-aligned vessels were used, many complaints/comments were made regarding the smell. The improvement in odour management may also have been due to the improved biofilter and therefore this combination of completely sealed lids and a compost/woodchip biofilter comes highly recommended.

#### *5.3.5. Vessel emptying*

Vessels are emptied by a suction pump. The majority of waste removed is in the liquid phase with the solid material settling at the bottom of the vessel. Although some of this material will continue to be broken down, eventually this will need emptying. Adkin et al. (2010) reported that prions would most likely be present in the solid fraction of the bio-reduced material. Bacteria and viruses would also be expected to adhere to the organic particulate matter (Beal et al., 2005; Rao et al., 1984). The storage of waste reduces pathogen loadings (Avery et al., 2005) but given that bio-reduction is currently operated under a continuous system, it is unlikely that wastes will be stored for a sufficient amount of time to reduce pathogens before fresh carcasses are added. The addition of lime (CaO) to waste material has shown to reduce pathogen loading (Avery et al., 2009) and therefore it is recommended that lime is added to bio-reduction vessels at a rate of 10 g l<sup>-1</sup> liquor (Avery et al., 2009) prior to the emptying of solid material. However, lime application to bio-reduction waste specifically has not yet been validated and requires further research.

Vessels were emptied and disinfected in between field trials in Article 3. Each vessel was disinfected using commercial strength hydrogen peroxide. Nevertheless, virus particles remained in the previously inoculated vessels and were isolated in background samples of the following trial (Article 3). As the virus in question was porcine parvovirus and the carcasses used were sheep, it was unlikely that these were natural populations. Virus particles can adhere to surfaces and can remain infectious for significant periods of time (Terpstra et al., 2007). Therefore, it is hypothesised that as the liquor level decreased due to evaporation, the viruses adhered to remaining organic material stuck onto the inner surface of the bio-reduction vessel. Once water levels were increased, the viruses were resuspended by the mechanical action of the aeration (Article 3). In this case the viruses survived as the bio-reduction vessels were operated under simulated breakdown procedures. It is envisaged that they would have continued to decrease in numbers and infectivity as they had in the previous trial had the vessels been operating under standard or optimal conditions.



### *5.3.6. Batch versus continuous input systems*

As there were various arguments in favour of and against the two different vessel designs, so are there arguments associated with using batch or continuous input systems. For example, wool is more recalcitrant than soft tissue and can remain in the vessel for long periods of time. It is also able to clog up the suction pumps that remove the waste. This can be problematic for continuous input systems where fresh inputs of wool are continually added. Therefore, it may be necessary to wait until the wool has decomposed before emptying the vessels. This may not always be feasible in continuous flow systems and therefore, it is recommended that vessels are emptied during periods of low mortality when all visible carcasses have degraded, such as between lambing seasons for sheep, before the vessels quickly fill. This problem obviously did not occur for the pig carcasses (Article 6) and therefore in this case, continuous input may be appropriate.

It takes approximately 28 days for whole carcasses (both sheep and pig) to degrade sufficiently to provide space for further inputs of carcasses (Article 3; Williams et al., 2009). Therefore, batch systems may be useful (i.e. two or more vessels) for larger farms where mortalities are constantly generated. However, on farms with low mortality, the use of one vessel with continuous input of carcasses may be more applicable. Williams et al. (2009) showed that one vessel had sufficient capacity to bioreduce all mortalities generated from a farm with a breeding ewe flock of 1,600. It is unlikely that farms of flock sizes smaller than this would benefit from bio-reduction due to the costs associated with running the bio-reduction vessels, unless vessels are switched off in periods of low mortality (Article 5). One problem envisaged for this action is the shock loading of the system when the vessels are brought back into commission, (section 5.3.2) further outlining the need for future research into the impacts of foaming. However, smaller vessels may be designed and this is an area that warrants further investigation. A preliminary survey of the farming community in North Wales suggested that farmers would be more likely to use bio-reduction if it was a communal asset (Gwilym, 2012) which may also make bio-reduction a financially viable option for farmers.

## 6. Conclusions

Bioreduction shows significant potential as a novel method of fallen stock storage prior to ultimate disposal. Pathogens were shown to not proliferate; indeed most of the pathogens tested significantly reduced in number in line with EFSA guidelines. However, the potential for pathogen release during foaming events is an area for concern that warrants further investigation. The biggest threat to the uptake of bioreduction, besides the reliance on the EFSA to approve this technology, is the cost to run the vessels. It is estimated that bioreducing 2.82 tonnes of carcasses a year would cost between £70,700 and £118,800 over the 25-year lifespan of the vessels. Reducing the electricity consumption would not only reduce this cost but would also reduce the carbon footprint. Improvements to the design of the vessels could be achieved through collaboration with businesses that are experienced in designing similar vessels (e.g. septic tank, in-vessel composting or anaerobic digestion designers). This would result in a system that is biosecure, practical and economically viable for industry to use, given a positive change in the EU regulatory framework.

## 7. Future work

As bioreduction is still such an emerging technology, there is a plethora of necessary and significant research that should be considered going forwards. A summary of potential areas to explore is given below.

### *7.1. Bioreduction as a disposal option*

- The fate of prions in bioreduction liquor and solids
- The fate of vegetative bacteria and protozoa in bioreduction liquor and solids using reverse transcriptase real-time PCR
- The fate of common pathogens associated with particular animal species, e.g. Porcine Reproductive and Respiratory Syndrome Virus (PRRSv) in pigs
- The environmental and biosecurity considerations of land-spreading bioreduction liquor

- The fate of pathogens in bioreduction liquor; including the ability of pathogens to enter a viable but non-culturable state during the bioreduction process and the potential to reactivate if spread to land
- The use of lime to reduce pathogen loading and to improve the liquor as a fertiliser
- The survival of pathogens during composting of the solid material

### *7.2. The practicalities of bioreduction*

- The efficacy of various disinfectants at cleaning bioreduction vessels
- The effect of reduced aeration rate and/or temperature on decomposition efficiency
- The effect of reduced water usage and electricity consumption on bioreduction efficiency
- Design review of the bioreduction vessels, specifically the need to:
  - a) Reduce electricity consumption by changing to a boiler-type vessel;
  - b) Improve insulation/thermal efficiency;
  - c) Improve electrical wiring;
  - d) Develop mechanical mixing methods;
  - e) Reduce foaming whilst keeping the vessels aerobic, and
  - f) Develop the ability to vent noxious gases before opening the vessels.

### *7.3. Developing a starting inoculum*

- The determination of the major bacterial and fungal organisms involved in the bioreduction process, more specifically those involved in the initial breakdown of carcasses
- The isolation of novel enzymes involved in the bioreduction process
- The development of a starting inoculum to increase the rate of bioreduction

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## **Appendix I:** The quantity of carcass components used in Article 2

The final quantity of water and carcass components used in the trial is based on the 300 kg of sheep and size of vessels used in a previous field trial (Williams et al., 2009) where:

- Bio-reduction Vessel (BV) capacity = 6,500 l
- Quantity of sheep carcass = 300 kg
- Quantity of water = 2,800 l
- Quantity of commercial accelerant = 1 g/kg carcass

The proportion of the mini bio-reduction vessel (MBV) to the BV is 0.000769

$$300 \text{ kg of meat} = (0.000769 * 300) = 0.231 \text{ kg}$$

$$= 231 \text{ g}$$

$$2,800 \text{ l of water} = (0.000769 * 2,800) = 2.15 \text{ l}$$

$$= 2153 \text{ ml}$$

Commercial accelerant dosing is 1g/kg, therefore  $0.231 \text{ kg} = 0.23 \text{ g}$

Under normal circumstances, the vessels will retain a proportion of the liquor from previous inputs in order to seed the vessels with the microbes and enzymes required to breakdown the carcass material. Therefore inoculum from a previous bio-reduction field trial was added in similar proportions to the quantity of gastrointestinal tract.

GI tract = 51 g in the MBV, therefore inoculum = 51 g.

As it was not possible to get a carcass small enough to fit into the 5 L MBVs, sheep carcass components were made up from various organs and viscera obtained from a local butchers and abattoir. In order to maintain a high particle size to mimic the carcass, a lamb chop was used to represent the bulk of the meat (Table AIa). Table AIb shows the ideal quantity of components to be used in the trial, along with the actual quantity of components.



**Table A1a:** The proportions of carcass components used in Article 2. These were based on a report of animal by-products by the MLC (2006). Components in italics were derived from commercially available lamb chops, other components were obtained from a local abattoir.

Carcass component	Weight (kg)	Component proportion
<i>Lean</i>	11.7	0.34
<i>Bone</i>	3.58	0.10
<i>Fat</i>	5.98	0.17
<i>Pelt</i>	1.99	0.06
Blood	1.93	0.06
Gastro-intestinal tract	7.6	0.22
Wool	0.97	0.03
Liver	0.69	0.02
Total weight	34.44	1
Proportion not used in trial	7.71	

**Table A1b:** Ideal and actual carcass components required for the laboratory-scale pathogens trial in Article 2. Component weights were calculated using the equation: Proportion of component (Table A1a) \* 231 g total weight. Where MBV = mini bioreduction vessel and CONBV = control mini bioreduction vessel.

Rep No	Chop (g)	Blood (g)	GI tract (g)	Wool (g)	Liver (g)	Total (g)
<i>Ideal</i>	<i>155.95</i>	<i>12.95</i>	<i>50.98</i>	<i>6.51</i>	<i>4.63</i>	<i>231.00</i>
MBV1	154.39	13	50.98	6.51	4.67	229.55
MBV2	154.82	13	51.08	6.51	4.64	230.03
MBV3	155.18	13	50.91	6.51	4.66	230.26
CONBV1	155.78	13	51.09	6.51	4.66	231.04
CONBV2	154.48	13	50.93	6.51	4.66	229.58

**Appendix II:** The physicochemical parameters analysed during the pathogen inoculation trials (Articles 2 and 3).

The physicochemical parameters were recorded during the bioreduction of sheep carcass components in the laboratory-scale study in Article 2 and during the field study run under standard operating conditions in Article 3. These parameters can be found in Table AIIa and Table AIIb respectively.

**Table AIIa:** The physicochemical parameters recorded during the bioreduction of sheep carcass components in Article 2. Where, MBV = mini bioreduction vessel, CONBV = control mini bioreduction vessel.

Parameters	Mini Bioreduction Vessels				Controls		
	MBV1	MBV2	MBV3	Mean	CONBV1	CONBV2	Mean
pH	7.13 ± 0.11	7.62 ± 0.17	7.32 ± 0.10	7.35 ± 0.14	7.25 ± 0.11	6.97 ± 0.17	7.11 ± 0.14
Temperature (°C)	23.6 ± 3.2	23.2 ± 3.5	22.9 ± 3.5	23.2 ± 0.2	22.9 ± 3.7	21.7 ± 2.9	22.4 ± 0.5
EC (mS cm <sup>-1</sup> )	2.5 ± 0.7	2.5 ± 1.2	3.2 ± 1.0	2.7 ± 0.2	1.5 ± 0.6	0.9 ± 0.2	1.1 ± 0.3
Moisture content (%)	99.0 ± 0.4	99.4 ± 0.2	99.4 ± 0.2	99.3 ± 0.2	98.9 ± 0.1	99.2 ± 0.2	99.1 ± 0.1
Organic matter of the dry weight portion (%)	63.1 ± 21.6	56.4 ± 15.8	55.4 ± 18.1	58.3 ± 9.4	75.1 ± 18.7	77.8 ± 17.1	76.4 ± 11.3
Total C (mg l <sup>-1</sup> )	1283 ± 187	690 ± 133	1004 ± 650	1030 ± 204	2298 ± 1142	1563 ± 876	1930 ± 664
Total N (mg l <sup>-1</sup> )	330 ± 98	135 ± 7	366 ± 252	295 ± 81	476 ± 189	234 ± 127	355 ± 115
NH <sub>4</sub> <sup>+</sup> (mg N l <sup>-1</sup> )	230 ± 86	76 ± 43	241 ± 177	196 ± 70	251 ± 94	57 ± 45	154 ± 64
NO <sub>3</sub> <sup>-</sup> (mg N l <sup>-1</sup> )	0.22 ± 0.05	-0.09 ± 0.06	0.13 ± 0.17	0.11 ± 0.07	0.24 ± 0.20	0.23 ± 0.26	0.23 ± 0.15
PO <sub>4</sub> <sup>3-</sup> (mg l <sup>-1</sup> )	2.42 ± 0.39	2.32 ± 1.19	3.02 ± 0.09	2.62 ± 0.29	1.85 ± 0.23	1.23 ± 0.16	1.54 ± 0.19
Na (mg l <sup>-1</sup> )	296 ± 25	408 ± 176	273 ± 81	315 ± 48	198 ± 24	96 ± 30	147 ± 29
K (mg l <sup>-1</sup> )	262 ± 49	327 ± 166	307 ± 95	295 ± 48	137 ± 18	75 ± 27	106 ± 20
Ca (mg l <sup>-1</sup> )	44 ± 10	66 ± 31	45 ± 18	50 ± 10	28 ± 5	14 ± 4	21 ± 4

**Table AIIb:** The physicochemical parameters recorded during the bioreduction of sheep carcasses under standard operating conditions in Article 3. Where, BV = bioreduction vessel, CONBV = in control bioreduction vessel and – = sample not taken.

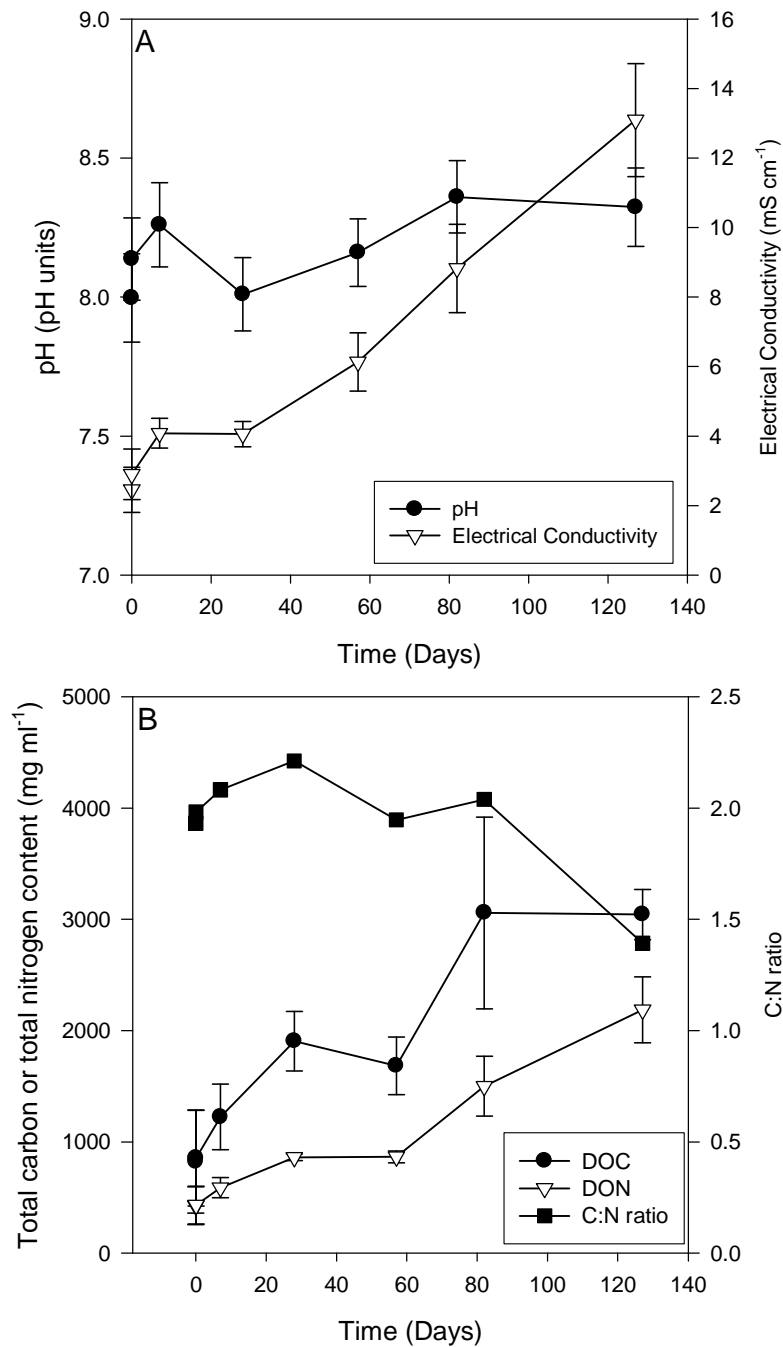
Parameters	Bioreduction vessels				Biofilter	Controls		
	BV1	BV2	BV3	Mean	Average	CONBV1	CONBV2	Mean
pH	8.24 ± 0.04	8.29 ± 0.10	8.01 ± 0.09	8.18 ± 0.05	7.24 ± 0.06	7.42 ± 0.12	7.96 ± 0.11	7.69 ± 0.10
Temperature (°C)	22.3 ± 3.3	30.1 ± 3.3	31.6 ± 1.9	28.0 ± 1.8	21.6 ± 1.8	8.9 ± 2.2	33.0 ± 1.3	22.8 ± 4.5
EC (mS cm <sup>-1</sup> )	5.3 ± 1.3	7.4 ± 1.7	5.2 ± 1.1	5.9 ± 0.8	0.5 ± 0.01	5.4 ± 0.6	9.9 ± 0.6	7.6 ± 1.7
Moisture content (%)	99.8 ± 0.1	99.6 ± 0.1	99.8 ± 0.1	99.7 ± 0.1	-	99.2 ± 0.7	99.5 ± 0.1	99.4 ± 0.3
Organic matter of the dry weight portion (%)	63.2 ± 3.0	77.5 ± 3.0	71.8 ± 3.3	70.9 ± 2.3	-	62.5 ± 8.0	69.0 ± 3.0	65.8 ± 4.2
Total C (mg/l)	1199 ± 352	2483 ± 408	1713 ± 393	1798 ± 242	780 ± 86	517 ± 236	4006 ± 680	2262 ± 628
Total N (mg/l)	864 ± 280	1217 ± 277	857 ± 207	979 ± 146	97 ± 16	331 ± 160	2048 ± 438	1189 ± 341
C:N ratio	1.39	2.04	2.00	1.84	8.04	1.56	1.96	1.90
NH <sub>4</sub> <sup>+</sup> (mg N l <sup>-1</sup> )	985 ± 301	1264 ± 409	859 ± 234	1036 ± 181	35 ± 8	448 ± 162	1724 ± 498	1086 ± 315
NO <sub>3</sub> <sup>-</sup> (mg N l <sup>-1</sup> )	0.30 ± 0.06	0.80 ± 0.30	0.46 ± 0.17	0.52 ± 0.12	0.85 ± 0.32	0.30 ± 0.07	0.34 ± 0.16	0.32 ± 0.08
PO <sub>4</sub> <sup>3-</sup> (mg l <sup>-1</sup> )	2.40 ± 0.24	2.69 ± 0.19	2.12 ± 0.28	2.40 ± 0.14	1.37 ± 0.15	2.32 ± 0.22	3.26 ± 0.22	2.79 ± 0.21
Na (mg l <sup>-1</sup> )	134 ± 41	175 ± 52	112 ± 21	140 ± 23	40 ± 5	93 ± 30	198 ± 45	145 ± 32
K (mg l <sup>-1</sup> )	211 ± 57	248 ± 66	162 ± 27	207 ± 30	379 ± 36	300 ± 92	300 ± 63	300 ± 53
Ca (mg l <sup>-1</sup> )	130 ± 20	156 ± 29	122 ± 19	136 ± 13	175 ± 11	84 ± 21	168 ± 19	126 ± 18
DO (mg l <sup>-1</sup> )	0.1 ± 0.06	0.3 ± 0.20	0.1 ± 0.05	0.2 ± 0.08	-	0.1 ± 0.05	0.5 ± 0.25	0.3 ± 0.14

## **Appendix III: Optimising the enzyme assay**

### **1. Introduction**

During bioreduction, animal carcasses are reduced to a nutrient-rich liquor over a period of three to six months; ultimately reducing the volume of waste to be disposed (Williams et al., 2009). The nutrient and physicochemical status of the liquor is governed by enzyme activity; these enzymes may be intrinsic to the animal carcass or provided by microorganisms. It is therefore possible that enzyme activity could be used to determine biological activity at any point in time, similar to how enzymes such as dehydrogenase are used to determine compost maturity (Tiquia, 2005). Determination of the most active enzymes will also help to enhance bioreduction by formulating an ‘accelerant’ that can catalyse the decomposition processes.

There are several hurdles to overcome when choosing which enzyme assays to use. Enzyme activity can be affected by a range of physical factors such as pH, temperature, organic matter content, particle size and salt content, to name a few (Gianfreda & Ruggiero, 2006). Some assays overcome these hurdles by analysing enzyme activity under optimal conditions, e.g. optimal pH. However, this doesn’t necessarily reveal anything about the activity of the enzymes *in situ* (Palmer & Bonner, 2007). During bioreduction, all of these factors are constantly changing as decomposition proceeds (Fig. AIIIa). Therefore, should an enzyme assay be optimised for any particular point in time, the assay conditions chosen may not be relevant further up/down the timeline. As a further complication, most enzyme assays used in this study have been developed for soil analysis and transcribing the assays for the nutrient rich bioreduction liquor may not be feasible. To overcome these obstacles, a series of enzyme assays were tested in the laboratory.



**Figure AIIIa:** The changing physicochemical parameters of the liquor during the bioreduction of sheep carcasses over 127 days; pH and electrical conductivity (EC) are shown in (A) and the dissolved organic carbon (DOC), dissolved organic nitrogen (DOC) and carbon to nitrogen ratio in (B).

**Table AIIIIa:** The hydrolytic enzymes assayed and their corresponding fluorescent substrates; MUF = methylumbelliferyl. Optimum incubation times using assumptions from Freeman et al. (1995) are included, i.e. only the linear section of the graph was used and allowing for 50 % error.

Enzyme	Enzyme classification	Substrate	Substrate supplier	Trend	R <sup>2</sup>	Linear section of first peak (min)	R <sup>2</sup>	Time optimum (min)	Substrate saturation (μM)
Acetylesterase	EC 3.1.1.6	MUF-acetate	Glycosynth, 44012	Polynomial	1.00	0 – 40	0.86	20	400
Acetylesterase	EC 3.1.1.6	MUF-butyrate	Sigma, 19362	Polynomial	1.00	0 – 40	0.92	20	400
Lipase	EC 3.1.1.3	MUF-nonanoate	Glycosynth, 44089	Linear	0.90	0 – 120	–	60	400
Phosphatase	EC 3.1.3.1	MUF-phosphate	Sigma, 69607	Polynomial	1.00	0 – 20*	–	20	900
Arylsulphatase	EC 3.1.6.1	MUF- sulphate potassium salt	Sigma, M7133	Linear	0.99	0 – 120	–	60	900
β-D-glucosidase	EC 3.2.1.21	MUF-β-D-glucopyranoside	Glycosynth, 44059	Linear	0.98	0 – 120	–	60	600

\*The first peak in fluorescence for MUF-phosphate was at 20 min. The 50 % error was omitted from this incubation time to allow for ease of measurements.

Proteolysis occurs at a non-uniform rate throughout decomposition (Forbes, 2008). Therefore it is hypothesised that protease activity will be recorded at most time points through the bioreduction process. The protease assay described originally by Ladd and Butler (1972) entails the use of casein as a non-specific substrate (Bonmati et al., 2009) and has been used in many soil studies to date (Carter et al., 2010; Rejsek et al., 2008). This paper uses the original assay as modified by Alef and Nannipieri (1995).

Six fluorescent hydrolytic substrates (Table AIIIa) were chosen to represent enzymes associated with the burial of carcasses in soil (Parkinson, 2009) as it is likely that these are also going to be present in the bioreduction vessels: arylsulphatases are involved in the cycling of sulphur (Li and Sarah, 2003); phosphatases in the cycling of phosphate, although the actual substrates are still unknown (Price and Stevens, 1999),  $\beta$ -D-glucosidase in the cycling of carbohydrates (Jeng et al., 2011; Knight and Dick, 2004), acetylsterases in the breakdown of acetylated polysaccharides (Basaran and Hang, 2000; Holmes and Masters, 1968) and lipases in the initial degradation of storage fats such as triglycerides (Cooper and Morgan, 1981).

Dehydrogenases are used in aerobic respiration by all microbes, thus making them a good general indicator of microbial activity (Alef and Nannipieri, 1995). There are two key dehydrogenase assays that have been used in soil studies; the reduction of 2,3,5-Triphenyltetrazolium chloride (TTC) or 2(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) (Islam et al., 2011; Taylor et al., 2002; Von Mersi and Schinner, 1991). Both methods induce a colorimetric change which can be measured using a spectrophotometer. Whilst a search of the literature has failed to turn up any studies relating to dehydrogenase activity during carcass decomposition *per se*, dehydrogenase activity is used to determine compost maturity by indicating a lower biological activity during the compost maturation phase (Barrena et al., 2008). As composting also involves the degradation of organic matter (Gwyther et al., 2011), the standard TTC method used by the US Department of Agriculture and US Composting Council (2001) has been chosen for this trial

## **2. Methods**

### *2.1. Protease assay*

#### *2.1.1. Liquor collection*



Liquor was collected from the simulated breakdown trial in Article 3 stored at 4-6 °C until used. Liquor was acclimatised to 40 °C before assays commenced. Samples were analysed according to Alef and Nannipieri (1995) with modifications by Rejsek et al. (2008).

### *2.1.2. Optimising assay conditions*

A 1 ml sample from each BV was added to a 50 ml centrifuge tube for each time point: 30, 60, 90 and 120 minutes. A second set of liquor samples was used as controls. To each of the 'test' tubes 5 ml of distilled water and 5 ml of 2 % sodium caseinate were added. The tubes were sealed and incubated shaking at 150 rev min<sup>-1</sup> in the dark at 40 °C according to the incubation times noted above. At the end of the incubation period 5 ml of 15 % trichloroacetic acid (TCA) was used to stop the reaction. For the controls, the sodium caseinate was added at the end of the incubation period prior to the addition of TCA. All samples were centrifuged at 10,000 rev min<sup>-1</sup> for 10 minutes. 5 ml of supernatant was mixed with 7.5 ml of alkaline reagent and the solutions incubated for a further 15 min at room temperature. After adding 5 ml of 33 % Folin-Ciocalteu reagent, the solutions were filtered and the absorbance measured after 1 hour at 700 nm. Standards were prepared using tyrosine giving final concentrations of 0, 33, 67, 100 and 167 µg ml<sup>-1</sup>. Briefly, tyrosine stock solution was added to 5 ml sodium caseinate and made up to 10 ml with distilled water. 5 ml TCA was added to each standard and treated as per the samples above. Optimum incubation times were determined by plotting absorbance against the amount of tyrosine measured.

A similar method was used to determine substrate concentration. However, instead of different incubation times the following concentrations of sodium caseinate were used: 0.5, 1, 2 and 4 %. Samples were incubated for 120 minutes. Optimum substrate saturation was determined by plotting the tyrosine concentration against substrate concentration.

## *2.2. Fluorescent substrate assays*

### *2.2.1. Liquor collection*

A starting inoculum was obtained by taking ~ 570 ml of frozen liquor from the simulated breakdown field trial outlined above, spanning all time points and all sample points. Liquor samples were defrosted, pooled and homogenised thoroughly by mixing in an orbital shaker (150 rev min<sup>-1</sup>, 40 °C). Approximately 300 ml of the homogenised liquor was

used as the inoculum. Pork (~ 100 g) and water (~100 ml) were added to the liquor and incubated as above for forty eight hours before samples were removed for analysis. A supply of pork liquor was maintained by routinely adding 100 g of pork (either diced pork or lean from pork chops) and 100 ml of water when the liquor was reduced to ~300 ml.

### *2.2.2. Optimising assay conditions*

The substrates listed in Table AIIIa were used to represent hydrolytic enzymes. Substrates and methylumbelliferone standard (Sigma Aldrich, M1381) were dissolved in 2 ml Methylcellosolve (Sigma Aldrich, 284467) for at least 2 hours before being made up to volume with distilled water. Acetylcetase substrates were dissolved in 5 ml methyl Cellosolve to try and improve solubility. Of the acetylcetase substrates only MUF-acetate was soluble at low concentrations. Both MUF-butyrate and MUF-nonanoate were therefore mixed thoroughly before use to produce a homogenised emulsion. All liquor, substrates and standards were equilibrated at 40 °C before use.

#### *2.2.2.1. Incubation times*

An arbitrary substrate concentration of 500  $\mu\text{M}$  was used for the MUF- $\beta$ -D-glucopyranoside, MUF-phosphate and MUF-potassium sulphate salt assays. Due to the insoluble nature of the acetylcetase substrates a lower concentration of 400  $\mu\text{M}$  was used. Each substrate was treated as follows. Six sets of 15 ml centrifuge tubes ( $n=5$  for each time point) were labelled with different incubation times; 0 min, 20, 40, 60, 90 and 120 min. To each tube 1 ml of liquor and 7 ml of substrate was added and the tube inverted 10 times to ensure thorough mixing. The samples were incubated at 40 °C in the dark to represent conditions within the bioreduction vessels. At the corresponding incubation time, 1.5 ml of sample was taken from each tube and centrifuged at 10,000  $\text{rev min}^{-1}$  for 5 minutes. supernatant (300  $\mu\text{l}$ ) was placed into a black 96 well plate and read in a Varian Cary Eclipse Spectrophotometer (Varian Inc., Palo Alto, CA) set to 450 nm emission and 330 nm excitation wavelengths with a slit width of 2.5 (Freeman et al., 1995). Fluorescence was plotted against time using Excel to obtain time curves. The optimal incubation time was determined using assumptions from Freeman et al. (1995) i.e. only the linear section of the time curve was used, allowing for 50 % error, and only short incubation times were used to reduce interference from the microbial growth.

#### 2.2.2.2. Substrate concentration

Once the optimum incubation time was determined each substrate was assayed using different substrate concentrations. The acetylcholinesterase substrates were measured at 0, 100, 200, 300 and 400  $\mu\text{M}$  whilst the remaining substrates were analysed using 0, 50, 100, 150, 300, 500, 800 and 1,000  $\mu\text{M}$ . Standards were prepared using 4-Methylumbelliferone with working concentrations of 0, 120, 240, 360, 480 and 600  $\mu\text{M}$  MUF. 7 ml of distilled water was added to each of 6x 1 ml liquor samples. Samples were inverted and centrifuged as per above. 250  $\mu\text{l}$  of each supernatant was added to wells in the 96 well plate. 50  $\mu\text{l}$  of standard was added to each well to give final concentrations of 0, 20, 40, 60, 80 and 100  $\mu\text{M}$  MUF. Fluorescence readings of the samples were compared to the standard curve before being converted into  $\mu\text{M}$  MUF released  $\text{min}^{-1}$  and plotted against substrate concentration. Optimum substrate saturation was taken as the substrate concentration that showed the highest activity.

#### 2.3. Dehydrogenase Assay

The dehydrogenase assay used was obtained from the US Department of Agriculture and US Composting Council (2001). As these are standard conditions used to measure dehydrogenase activity in compost we have kept the conditions the same with the exception that 3 g of compost is replaced with 3 ml of liquor. Briefly, the liquor (see section 2.2.1), 3 ml of distilled water and 3 ml 3% 2,3,5-Triphenyltetrazolium chloride (TTC; Sigma Aldrich, T887) were mixed for 30 s and incubated for 24 h in the dark at 37 °C ( $n = 4$ ). A control for each replicate was prepared by adding 6 ml water to 3 ml liquor and a blank without liquor was prepared by mixing 3 ml distilled water and 3 ml TTC. Both the control and blank were incubated as above. After 24 hours, 10 ml of methanol was added to each sample and mixed for 5 min at 200  $\text{rev min}^{-1}$  before the suspensions were filtered through a glass fibre filter into fresh containers. Methanol was used to wash the red colour from the glass fibre filter and the filtrate collected and made up to 100 ml with methanol. Three 300  $\mu\text{l}$  aliquots of each sample were transferred to a 96 well plate and absorbance was read at 485 nm. A standard curve was generated using 1,3,5-Triphenyltetrazolium formazan (TPF; Sigma Aldrich, 93145) dissolved in methanol. Final values were expressed as mg TPF released  $\text{l}^{-1}$  liquor  $\text{min}^{-1}$ .

### 3. Results

#### 3.1. Protease assay

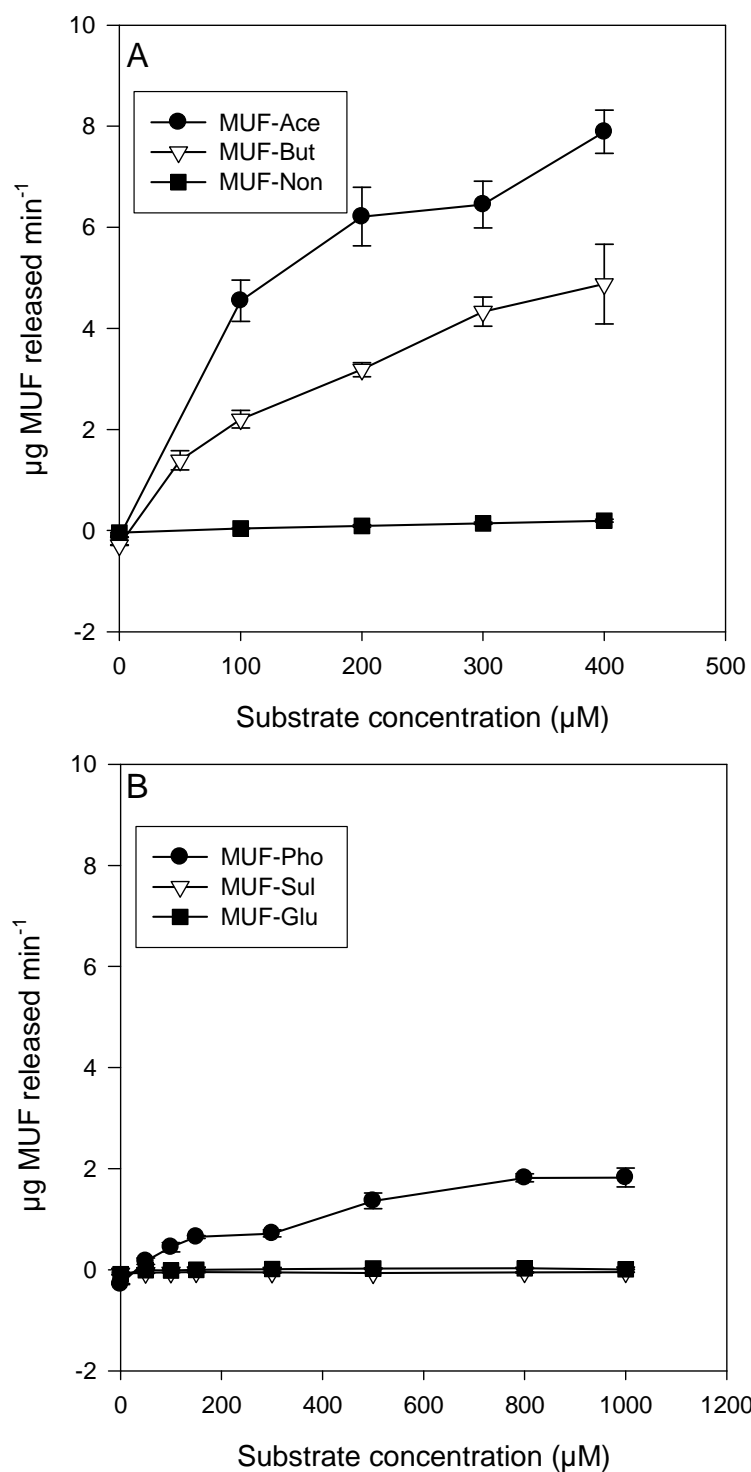
The optimum incubation time for the protease assay was 120 minutes. Substrate saturation showed a negatively linear trend with the greatest absorbance values at 0.5 % sodium caseinate ( $R^2 = 0.948$ ). However, all absorbance readings were lower than the absorbance values for  $0 \mu\text{g ml}^{-1}$  tyrosine standard (data not shown).

#### 3.2. Fluorescence assays

Optimum incubation times varied depending on the substrate used (Table AIIIa). MUF-acetate, MUF-butyrate and MUF-phosphate all showed two peaks in activity; at 40 and 120 min for MUF-acetate and MUF-butyrate and 20 and 90 min for MUF phosphate. A linear trend was obtained with MUF-nonanoate, MUF-sulphate and MUF-glucopyranoside with maximum fluorescence at 120 min. Optimal substrate concentrations for all three acetylerase substrates was  $400 \mu\text{M}$  (Fig. AIIIb). MUF-phosphate had an optimum concentration of  $900 \mu\text{M}$  and MUF-sulphate an optimum of  $900 \mu\text{M}$  whilst MUF-glucopyranoside was slightly lower at  $600 \mu\text{M}$  (Fig. AIIIb).

#### 3.3. Dehydrogenase assay

The average activity was  $0.15 \text{ mg TPF ml}^{-1} \text{ liquor min}^{-1}$ .



**Figure AIIIb:** Substrate concentration curves for the following fluorescent substrates: MUF-ace = 4-Methylumbelliferyl acetate; MUF-But = 4-Methylumbelliferyl butyrate; MUF-Non = 4-Methylumbelliferyl nonanoate; MUF-Pho = 4-Methylumbelliferyl phosphate; MUF-Sul = 4-Methylumbelliferyl sulphate potassium salt and MUF-Glu = 4-Methylumbelliferyl glucopyranoside.

#### 4. Discussion

The results of the protease assay gave negative results due to the control samples having similar colorimetric readings to the test results. This suggests that there was either very little protease activity or inversely, that protease activity was saturated and so the addition of fresh substrate did not increase the activity any further. The latter is probably more accurate considering the nature of decomposition. However, whatever the cause for the negative results, it is obvious that the TTC method of Alef and Nannipieri (1995) is not suitable for bioreduction analysis. Gianfreda (2006) outlines the pros and cons of fluorescence assays using methylumbelliferyl substrates as compared to colorimetric assays. Some of the benefits of these fluorescence substrates apply to protease assays using fluorescein isothiocyanate (FITC) casein as the substrate. For example, assays can be done in 96-well plates with small amounts of sample and there is no need to stop the reaction prior to measurement. The FITC casein is quenched and is only released when protease activity digests the casein into smaller peptides (G-Biosciences, 2011). Therefore, a commercial protease assay using the FITC substrate will be used in future experiments (G-Biosciences, 2011).

A linear relationship between incubation time and enzyme activity was not observed for all of the fluorescent substrates (Table AIIIa). Therefore the linear portion of the first peak was used to generate optimal incubation times. All substrates, with the exception of MUF-sulphate, approximate the Michaelis-Menten model for single substrate reactions (Fig. AIIIb). Despite showing the standard Michaelis-Menten model in peat (Freeman et al., 1995), in pig liquor MUF-sulphate showed two peaks in activity; the first at 200  $\mu\text{M}$  and the second at 900  $\mu\text{M}$ . This effect is masked in Fig. AIIIb due to the low activity of sulphatase in the pig liquor. A substrate concentration of 900  $\mu\text{M}$  has been chosen for the sulphatase assay to ensure complete saturation as assays are more reliable with higher starting substrate concentrations (Palmer and Bonner, 2007). The greatest hydrolase activity was observed in the acetylcholinesterase substrates; MUF-acetate and MUF-butyrate (Fig. AIIIb), which can be explained by the high fat content in pork. Both acetylcholinesterase substrates have been criticised as being unspecific substrates for lipases (Cooper and Morgan, 1981) and therefore have been chosen to represent esterases. The results from this trial also suggest that the two substrates were not specific for lipases as the MUF-nonanoate assay showed very little activity in comparison. Although unspecific, all three substrates give a good overall indication of the breakdown of lipids within the pork substrate.

The plethora of methods used to measure and record dehydrogenase activity makes comparison of results difficult, particularly as other waste streams are generally measured in g dry weight<sup>-1</sup> (Barrena et al., 2009; Benito et al., 2003; Zeng et al., 2010). Nevertheless, this data supports the theory that the mesophilic, aqueous environment is conducive to increased microbial activity and that microbial activity is a key driver in bioreduction.

Despite attempts to monitor actual rather than potential enzyme activity the need to use the commercial protease kit outweighed the benefits of investigating and perfecting other assays. Similarly, the dehydrogenase assay followed standard conditions for the composting industry. Nevertheless, the dehydrogenase does not use a buffer, is kept at the pH of the liquor itself and the incubation temperature is not far off that of the bioreduction vessels, therefore, activity will closely reflect that which is found in the bioreduction vessels. The fluorescent assays are designed to measure actual activity. However, these assays may be influenced by the pH of the liquor, the salt content and the organic matter content (Gianfreda & Ruggiero, 2006) and therefore, the results should be considered indicative rather than absolute.

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