Final Report

Impacts of pasteurisation and mesophilic AD on some common crop pests and diseases in the UK

The susceptibilities of a number of common agricultural pests and diseases (including blackgrass, clubroot and late blight) to pasteurisation and/or mesophilic anaerobic digestion were investigated through literature review and laboratory experiments.

The results of the experiments are reported here, and indicate that survival rates were all low, although responses to different treatments varied amongst the organisms tested.

This report is of particular relevance to those seeking to understand the quality and safety of digestates from farm-fed AD facilities.

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Front cover photography: Crop residues as feedstock for an AD process

Executive summary

It is recognised that AD is an important means of recovering value from many biodegradable wastes, where their production cannot be avoided. AD is an effective source of renewable energy as well as a potentially useful biofertiliser in the form of the digestate. While the use of digestate as a fertiliser can offer significant benefits, it is important to ensure that the digestate is safe for use.

Previous research has examined the impacts of AD on a range of common pests and diseases, and the risks from these are thought to be low, particularly for AD systems that incorporate a discrete pasteurisation step. This project was initiated to confirm and add to published data – and is expected to be particularly useful for those operating farm-fed AD systems that do not incorporate a discrete pasteurisation step.

The survival of a range of plant pathogens and weed species was determined in experimental AD systems. The organisms chosen for the study were designed to cover a range of those that might commonly be encountered in full scale AD systems, particularly those accepting feedstocks that include manures and crop residues:

- Weed seeds (known to survive in seed banks for many years), including Blackgrass;
- Protists with hardy resting spores that are known to survive in soil for c. 20 years, including Clubroot of brassicas; and
- Fungi with a range of resting spore types, including some examples of the Fusaria that are known to produce mycotoxins harmful to human and animal health.

Although not expected to be present in AD feedstocks, the impacts of AD processing on a number of bacteria designated as quarantine diseases of potato were also examined.

Inocula of these organisms were produced to reflect the most relevant spore type or other structures normally encountered in food or crop wastes. Feedstocks consisted of sterilised food and vegetable wastes, and the experimental batch and/or semi-continuous AD systems were primed with digestate from a commercial food digester. Once acclimated, the reactors were spiked with microbial species or seeds, the survival of which was then monitored. Spikes were introduced at high levels to facilitate detection – and to provide a worst-case scenario for any infected material entering a commercial AD system.

The processes examined included pasteurisation (at 70°C for one hour), mesophilic anaerobic digestion (MAD) at 37.5°C, and digestate storage at 7-11°C (for which the test organisms were spiked into the stored digestate). A combination of batch and semi-continuous AD was used, with two organisms tested in both systems (tomato seed and *F. culmorum*). Experimental and quarantine constraints meant that not all organisms could be tested in both systems.

Detection methods used to verify survival of test organisms were designed, where possible, to ensure that viability was confirmed as well as presence.

Pasteurisation was, as expected, shown to represent an effective treatment for reducing the survival of most pathogens and seeds tested. The most resilient organism was found to be *Spongospora subterranea,* which is well known to survive for long periods as cystosori and to be difficult to eradicate. The similar organism, *Plasmodiophora brassicae,* appeared to be less resilient. Tomato and blackgrass seed also proved to be resilient, surviving up to 5-6 days in MAD at 37.5°C, although they did not survive pasteurisation. The common indicator pathogens (*E. coli* and *Salmonella* Typhimurium) were both eliminated by pasteurisation, and reduced by MAD. Organisms which were tested in both systems (tomato seed and *F.*

culmorum) showed similar but not identical results indicating that there is some variation between the systems.

Time after which levels of inoculated organisms dropped below detection limits (LOD). Batch AD results

Organism	Pasteurisation 70°C	MAD 37.5°C	Stored in digestate at 7-11°C	Detection method(s)
Tomato seed (*Lycopersicum esculentum*) cv Ailsa Craig	1 hour	Still viable at 6 days	Still viable at 10 days	Tetrazolium staining
Black grass seed (*Alopecurus myosuroides*)	1 hour	5 days	Still viable at 10 days	Tetrazolium staining
Phytophthora infestans	1 hour	1 day	1 day	Culture
Phytophthora cinnamomi	1 hour	1 day	5 days	Culture
Phytophthora nicotianae	1 hour	1 day	5 days	Culture
Fusarium culmorum	1 hour	1 day	5 days	Culture
Fusarium oxysporum f. sp. radicis lycopersicae	1 hour	1 day	5 days	Culture
Plasmodiophora brassicae	1 hour	1 day	6 days	PCR, Bioassay, Bioassay+PCR

Time after which levels of inoculated organisms dropped below detection limits (LOD). Semi-continuous AD results

Organism	MAD + Pre-Pasteurisation	MAD	Detection method
Escherichia coli	1 hour	1.8 log decrease at 30 days	Culture
Salmonella	1 hour	3.5 log decrease at 30 days	Culture
Tomato seed (*Lycopersicum esculentum*) cv Ailsa Craig	1 hour	3 days	Germination
Streptomyces scabies	12 days	12 days	DNA PCR
Spongospora subterranea	3 days	12 days	Bait test+DNA PCR
Rhizoctonia solani	15 days	15 days	DNA PCR RNA PCR
Ralstonia solanacearum	6 days	6 days	BIOPCR
Clavibacter michiganensis subsp. *sepedonicus*	6 days	6 days	BIOPCR
Fusarium culmorum	3 days	3 days	DNA PCR RNA PCR

In summary, it is clear that in most cases the risk of organisms surviving AD processes is low, provided they are carried out at the temperatures and conditions prescribed under PAS110, including pasteurisation at 70°C for one hour. Options for alternative pasteurisation approaches are discussed in a separate report (OMK007-002S).

Contents

Glossary

AD	Anaerobic digestion – the process of controlled decomposition of biodegradable materials within an atmosphere devoid of free oxygen
CFU	Colony Forming Units – a measure of the size of a microbiological population
CHP	Combined Heat and Power – an electro-mechanical engine which generates both electricity and heat
Digestate	A by-product of the anaerobic digestion process which can be used in liquid form as an effective biofertiliser recycling nutrients back to land, and in solid (fibre, or cake) form as a soil conditioner
ELISA	Enzyme linked immunoassay – a biochemical test using antibodies to determine the presence of a target substance in a suitable sample
FIT	Feed in tariff, a UK government renewable energy incentive
GHG	Greenhouse gas(es)
MAD	Mesophilic anaerobic digestion – AD processes utilizing bacteria with optimal growth temperatures in the range 30 to 45°C
MPN	Most Probable Number – a measure of the size of a microbiological population
PAS110	Publicly Available Specification 110 – the UK's baseline specification for digestate quality
PCR	Polymerase chain reaction – a biochemical process that amplifies a single or a few copies of a piece of DNA to enable its detection
QP	Quality Protocol – a regulatory device that sets out end of waste criteria for specific waste-derived materials (such as digestate)
RHI	Renewable heat incentive, a UK government incentive to promote the use of heat from renewable sources
ROC	Renewable obligation certificate, issued for eligible renewable electricity generated within the UK
TAD or TAnD	Thermophilic anaerobic digestion – AD processes utilizing bacteria with optimal growth temperatures in the range 45 to 80°C
TaqMan PCR	Polymerase chain reaction method using TaqMan® technology

1.0 Introduction

At the time of writing, the number of digesters in use in the UK outside of the water industry is quite small, with 88 operational facilities as of November 2012 (NNFCC, 2012). But numbers are predicted to grow rapidly over the next decade with a range of financial incentives in place to encourage this growth.

Anaerobic digestion (AD) is a process where biodegradable plant and animal material (biomass) is converted into useful products by microorganisms in the absence of oxygen. Biomass is fed into sealed tanks and (either in parallel within a single digestion vessel, or in a series of separate vessels) naturally occurring microorganisms break down the biomass in discrete stages, ultimately releasing biogas. Biogas comprises a mix of methane, carbon dioxide and other trace gases that represents a source of clean renewable energy. The material left over at the end of the digestion process is rich in nutrients (ammonium nitrogen, phosphate and potassium) and is therefore an excellent replacement for some mineral fertilisers. This material is known as biofertiliser or digestate. Many kinds of biomass can be processed in an anaerobic digester, including: food waste, crops and crop residues, biosolids (sewage sludge), livestock slurries and manures. However, the acceptability of specific waste types will vary depending upon the regulations governing the AD process and intended biofertiliser use.

Its nutrient content means that digestate is usually recycled to agricultural land. This route offers many environmental benefits as it closes the nutrient cycle for nitrogen, as well as providing trace elements and a source of organic matter. As such it helps maintain and improve soil fertility and function.

In order to ensure the confidence of end-users for digestate and to encourage an active market in this material, a publicly-available specification (PAS), BSI PAS 110, was published in 2010 following consultation with end users, environmental regulators and the UK AD industry. This allows producers to demonstrate that their digestate is of consistent quality and fit for purpose.

This PAS requires that specific stages in the digestate production process are undertaken that will sufficiently control the risks associated with the use of digested materials. These stages include (with some exceptions) a requirement for inclusion of a pasteurisation phase within the overall AD process to control plant and animal pathogens to within acceptable tolerances. Whilst the inclusion of a pasteurisation phase is a statutory requirement where AD facilities are accepting Animal By-Products and other kinds of food waste for processing, this statutory requirement does not extend to AD facilities that only accept plant residues or a range of agricultural inputs for processing. Whilst the PAS requirement that even these materials be subjected to a pasteurisation phase offers reassurances on digestate quality to digestate users, it adds cost to digestate producers.

2.0 Aims and objectives

2.1 Aims of the study

Numerous previous reports and recent technical research carried out on behalf of WRAP suggest that mesophilic anaerobic digestion offers a robust mechanism for the production of safe, sanitised digestate, particularly when the mesophilic step is accompanied by batch pasteurisation. However, much of the evidence-base that has informed this technical work has been drawn from cross referencing to the time-temperature exposures common under lengthy, self-heating (aerobic) composting conditions. Given the wet conditions found in most AD processes and the consistent, controlled nature of heat applied during pasteurisation steps in such processes, it is reasonable to assume that the impacts of heat on pests and diseases of interest will be held in common between composting and AD treatments.

The aim of this project was to demonstrate empirically the robustness of this approach by examining the impacts of MAD with or without pasteurisation and digestate storage on a selection of pests and diseases potentially associated with plant materials that can be used as feedstocks for anaerobic digestion.

2.2 Objective of the study

To assess the impacts of wet, mesophilic anaerobic digestion systems in combination with pre or post-AD pasteurisation stages (or no pasteurisation), on a range of common agricultural pests and diseases.

3.0 Review of current practices and conditions in common UK AD processes

3.1 Introduction

Over the past decade the use of anaerobic digestion to treat and stabilise organic wastes has increased rapidly. This trend has been driven by the European Landfill Directive (99/31/EC) with its aim of diverting biodegradable wastes from landfill.

In addition there is a need to reduce the country's overall GHG emissions by utilising additional sources of renewable energy. At a European level, legislation has been introduced to set targets for renewable energy (EU Directive 2009/28/EC)[1] and limit global land conversion for biofuel production. EU Directive 2009/28/EC sets ambitious targets for all Member States, such that the EU will reach a 20% share of energy from renewable sources by 2020.

In the UK, incentives have been offered for the generation of renewable energy and heat in the form of renewable obligations certificates (ROCs), feed in tariffs (FITs) and renewable heat incentives (RHI)[2]. This is likely to drive the trend towards using AD as a means of recovering value from waste through renewable energy production.

The majority of commercial AD facilities in the UK use wet, mesophilic processes[3]. As well as biomethane, these digestion processes also generate digestate with a solids content of around 4% that can be dewatered to a cake (or 'fibre') of up to 30% dry solids. Digestate comprises the inert inorganic and the non-biodegraded organic fraction of the feedstock, together with microorganisms that grew during the process. Digestate is a potentially valuable resource that can be recycled to agricultural land as a biofertiliser. If used in this way it closes the nutrient cycle for nitrogen as well as providing a source of both trace elements and organic matter.

A wide range of human, animal and plant pathogens are known to occur in biodegradable waste materials, with the type and population of the pathogen dependent on the source of the material. Consequently there are understandable questions about potential contamination of crops, grazing animals and the food chain that might result from recycling digestate to agricultural land. To address possible risks to the environment, crops, animal and human health, the recycling of digestate is tightly controlled through both legislation and Codes of Practice (described below). These present a multi-barrier approach to minimize possible risks that includes: minimising the numbers of potential pathogens within the material for recycling; the adherence to specified processing parameters; restricting the potential applications for the end-product; and introducing a time-related grazing or harvesting ban on land where the material is recycled.

[1] *http://ec.europa.eu/energy/renewables/targets_en.htm*
[2] *http://www.biogas-info.co.uk/index.php/incentives-qa.html*
[3] *http://www.biogas-info.co.uk/index.php/types-of-ad.html*

Feedstocks derived from biodegradable materials other than raw sewage sludge are generally considered as wastes and as such any AD process accepting them as a feedstock would be covered by waste management regulations, as would the application of any resulting digestates on land[4]. However, AD processes accepting specific source-segregated biodegradable inputs may produce digestates that are eligible for inclusion within the Anaerobic Digestate Quality Protocol (ADQP) (EA, 2010). The ADQP defines the point where digestates derived from specified source-segregated biodegradable wastes can be considered products when supplied to specified markets. Such digestates can (for example) be supplied to farms for normal agricultural use without reference to waste management regulations.

The ADQP is intended to be used alongside the voluntary BSI PAS 110 (BSI, 2010), which provides the procedures, controls and monitoring necessary to meet the end-of-waste criteria for an acceptable standard. PAS 110 requires that digestates meet a range of quality limits, including PTEs (potentially toxic elements), stability and physical contaminants. For those digestates that are required to meet the requirements of the EU Animal By-Product Regulations (EU, 2009) there are statutory limits for *E. coli* and *Salmonella* spp. PAS 110 also prescribes limits for *E. coli* (maximum of 1,000 cfu/g fresh matter) and *Salmonella* (absent in 25 g fresh matter) for digestates that fall outside the scope of the Animal By-Product Regulations.

3.2 The Anaerobic Digestion Process

The majority of digesters built and operated in Europe and in particular in the UK are known as mesophilic anaerobic digesters (MAD), being operated in the mesophilic temperature range of ~35±3°C. Digesters operated at higher temperatures are known as thermophilic anaerobic digesters (TAD or TAnD) and this usually refers to an operating temperature of ~55°C. It is not uncommon for a digestion process to include one or more digesters operated in series, with the contents of the first digester passing directly to the second digester etc. There may be a number of good process reasons for doing this, for instance to reduce possible short-circuiting, or to operate each digester at different temperatures to enhance pathogen reduction. This is referred to as a staged process, for instance a two-stage digester. By contrast, some processes seek to separate the different phases of the actual digestion process by manipulating the operating conditions within each digester. For instance hydrolysis may occur in the first digester with acidogenesis and methanogenesis in the second. This is known as a phased process, such as acid phased digestion (APD). Wherever possible in this review, the distinction between each of these process variants is made clear. However it is frequently the case in the literature that while much information is provided as to the behavior of a given pathogen during digestion, very little information is provided on the digestion process itself.

The anaerobic digestion process itself is generally optimised for the destruction of volatile organic matter and the production of biomethane – rather than the destruction of pathogens. In addition to the anaerobic environment within the digester, operating temperatures of 35°C, the absence of predatory protozoa, and regular feeding provide conditions not dissimilar to those of the human and animal gut. Thus it does not pose a hostile environment for most enteric pathogens and is not necessarily an effective method for inactivation of many of the pathogens that might occur in feedstocks. As a result, other pre- or post-treatment processes are employed specifically to reduce pathogen numbers. Suitable pre- or post- treatment methods could be modified from other waste treatment processes (such as the stabilisation of digested sludge cake with lime, or aerobic composting of fibre digestates).

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[4] Note that digestates resulting from AD processes accepting only crops grown specifically for digestion, with or without livestock manures / slurries are not generally considered wastes (http://adlib.everysite.co.uk/resources/000/264/268/Anaerobic_Digestion_and_Environmental_Permitting.pdf)

3.3 Review of published literature

Prior to commencement of laboratory work a literature review was carried out, collating current knowledge of the fate of selected pathogens and seeds in AD systems – with and without pasteurisation stages. This review is reproduced in full in Section 8.0, with the following summary:

- The AD process may be more effective in eradicating a number of plant pathogens and weed seeds than equivalent levels of moist heat in soil, compost or water, possibly due to the release of toxic substances or low pH, depending on the feedstocks used for AD;
- The majority of plant pathogens examined were reduced to below detectable limits after periods of MAD of 28 days or less. However, there is conflicting evidence from different studies for some pathogens e.g. *P. brassicae* and *C. michiganensis* ssp. *michiganensis*. This may be due to different types of pathogen inoculum used or the conditions of the tests;
- Much of the information on the effects of AD on weed seeds is on species that are of concern in North America; information on some important weed seeds in the UK, e.g. black grass (*Alopecurus myosuroides*) is lacking;
- There is little information on the effects of short periods of high temperature, thermophilic AD on plant pathogen and weed seed eradication.

The experimental work carried out in this project was designed to add to existing data as well as fill some of the known data gaps, by investigating the survival of a range of pathogens with differing biological characteristics through AD processes.

4.0 Experimental methods

4.1 Experimental methodology: Batch culture AD system

Batch AD tests were conducted in bench-scale equipment consisting of two litre 'Quickfit' multiadapter flasks (Fisher Scientific, Loughborough, UK) immersed in thermostatically controlled water baths, each holding up to four flasks (Fayolle *et al.*, 2006).

The digestate mix in the flasks consisted of 67% w/w pasteurised food waste digestate from a commercial AD plant), homogenised fruit (9%), vegetable (8%), and bakery (4%) wastes and 12% water.

An exhaust pipe from the top of each flask headspace passed through a water air trap, enabling samples of gas to be analysed for ammonia, methane and hydrogen sulphide using gas detector tubes (Draeger, Lübeck, Germany). Typical operational conditions are presented in Section 9.0. The temperature of the digestate/food waste mix in each flask was continuously monitored with temperature probes and a data logger.

The following experimental treatments were applied to the contents of the flasks:

(a) Pasteurisation at 70°C for 1 hour, pre-MAD (mesophilic anaerobic digestion);
(b) MAD at 37.5°C for 10 days (without pasteurisation), with samples taken after 1 hour, 1, 5, 6 and 10 days;
(c) Pasteurisation at 70°C for 1 hour, post-MAD (if inoculum proved viable after 10 days MAD);
(d) Storage at 7-11°C for 10 days, with samples taken after 1hour, 1, 5, and 10 days.

Each flask contained sufficient numbers of sample bags of the specified organisms (seed type or pathogen isolate inoculum) for each of the specified exposure and sampling times. For seeds and for each of the other types of test organisms, two replicate flasks were prepared for each of the experimental treatments (a) to (d).

4.1.1 Test organisms

Samples of seeds or pathogen inoculum were filled into 50 mm diameter polyester mesh bags which were inserted for specified times into the experimental flasks, and then tested for viability. Two replicate batches of day 0 seeds or pathogen inocula were also tested to ensure that recovery methods were functioning correctly.

As a result of previous work for WRAP (for example Pollard *et al.,* 2008) and known literature gaps, the following test organisms were examined:

Tomato seed (*Lycopersicum esculentum*) cv. Ailsa Craig
Black grass seed (*Alopecurus myosuroides*)
Phytophthora infestans
Phytophthora cinnamomi
Phytophthora nicotianae
Fusarium culmorum
Fusarium oxysporum
Plasmodiophora brassicae

4.2 Experimental methodology: Semi-continuous model AD system

Four bench scale MAD reactors (2.5 litre top water level) (Figure 4-1) were acclimatised for 21 days, operating at a stable pH (6.8-7.5), low VFA: alkalinity ratio (<0.3), and producing good quality biogas (>55% methane). The reactors were fed (and decanted) on a daily basis with blended and diluted food waste at a dry solids concentration of 5.0% (Table 4-1). Once acclimated, the reactors were spiked with test organisms then operated for a further 30 days (2 minimum hydraulic retention times).

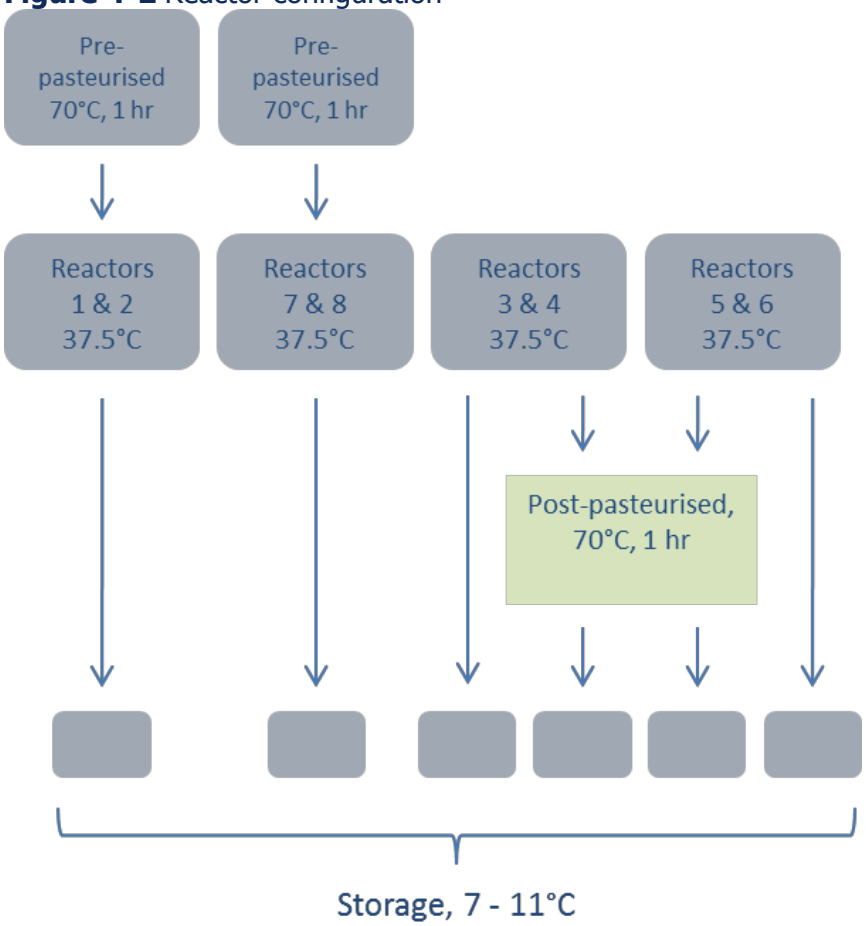
Table 4-1 Food waste composition (based on WRAP, 2010)

Category	% contribution on a wet weight basis
Dairy & egg	**8.3**
Meat & fish	**8.3**
Meals	**9.5**
Bakery	**11.9**
Fresh Fruit	**15.5**
Drink	**19.1**
Fresh vegetables & salads	**27.4**

Figure 4-1 Bench scale plant configuration showing MAD rigs and biogas collection

The reactor configurations were designed to examine the impact on test organisms of pre-pasteurisation (70°C for 1 hour) on the feedstock, MAD operation (37.5°C), pasteurisation post digestion (70°C for 1 hour) and storage (7-11°C) (Figure 4-2).

Figure 4-2 Reactor configuration



- The feedstock, based upon WRAP data (Table 4-1), was blended, autoclaved and diluted with distilled water to 5.0% dry solids;
- A proportion of the autoclaved mixture was then spiked with a known quantity of the test organisms, split into two samples, one being pasteurised;
- Reactors 1 & 2 were fed (1/15th of the working volume) with the pasteurised, and 3 & 4 with the non-pasteurised feedstock. A tracer (lithium chloride) was also added to the spiked feed to allow dilution to be monitored;
- For the following 30 days 1/15th of the reactor volume was removed on a daily basis and replaced with autoclaved feedstock (not spiked);
- The quantity of microbial species which died off was calculated by subtracting the number of organisms washed out from the process from the total number at the beginning of the trial and calculating the difference between that figure and the number measured at specified intervals (days 1, 2, 3, 6, 9, 12, 15, 20 & 30). The calculation method to determine die off rates and the tracer curve appear below (Section 4.2.1);
- The reactor contents at the end of the trial were then stored for 35 days at 5°C and levels of microbial species present measured at set intervals (days 0, 6, 13, 20 & 35).

The set of reactors (1, 2, 3 & 4) described in Figure 4-2 was set up at the Aqua Enviro site and inoculated with faecal indicator pathogens (*Escherichia coli* and *Salmonella* Typhimurium, NCIMB Aberdeen, culture number 10248). A duplicate set of reactors (5, 6, 7 & 8) was set up at APHA in the plant health quarantine facilities with inocula of plant pathogens only. Reactors 5 & 6 were fed with un-pasteurised feedstock, whereas reactors 7 & 8 were fed with pasteurised feedstock. As a result of previous work for WRAP (for example Pollard *et al.,* 2008) and known literature gaps, the following test organisms were examined at APHA:

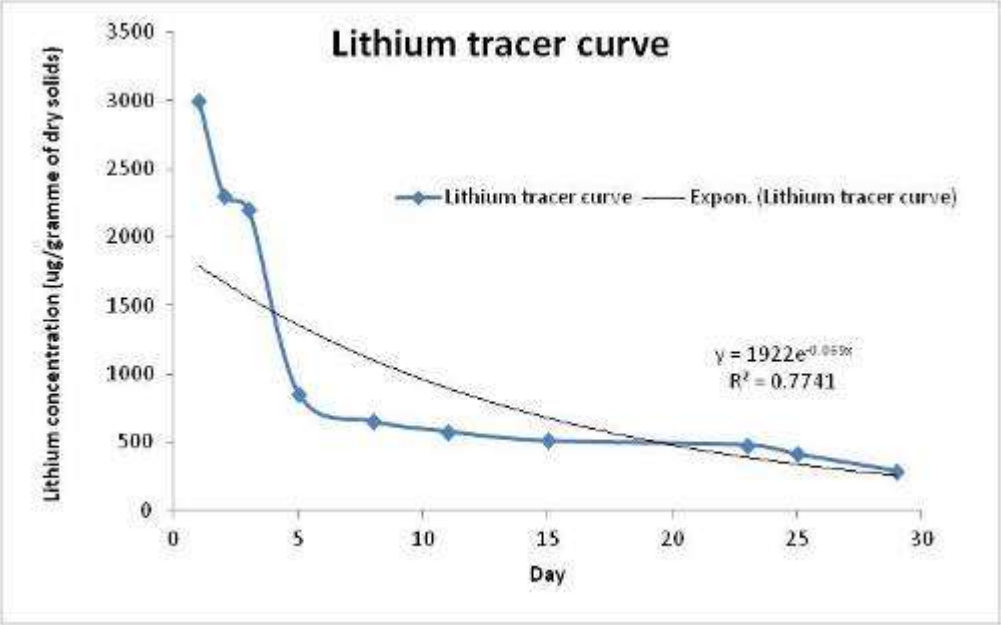
Tomato seed (*Lycopersicum esculentum*) cv. Ailsa Craig
Streptomyces scabies
Spongospora subterranea
Rhizoctonia solani
Ralstonia solanacearum
Clavibacter michiganensis subsp. *sepedonicus*
Fusarium culmorum

Tomato seed and *Fusarium culmorum* were common to both batch and semi-continuous AD experiments, allowing comparison between the two approaches.

4.2.1 Methodology for calculating for die-off and tracer curve (for E. coli)

1. After spiking and at every sample time after that, the concentration of lithium, of dry solids, and of test organism (for example *E. coli*) was measured;
2. The concentrations of *E. coli* in terms of MPN/g DS and the concentration of lithium in 1g DS were calculated at each sample time;
3. The lithium concentrations were used to derive a % remaining curve by dividing the amount of lithium present on any given day by the initial concentration;
4. Multiply the initial concentration of the test organism (*E. coli*) by the % remaining curve to determine the amount of test organism which has been washed out;
5. Convert this into a log value washed out;
6. Convert the measured test organism values into a log value;
7. Subtract the measured log value from the log washed out value to obtain the difference, which is the die off rate.

Figure 4-3 Lithium tracer curve, as measured



4.3 Inoculum production/sources for AD pathogen experiments, and detection methods

Table 4-2 shows the inoculum types used in the semi-continuous system and the detection methods used. More information on the reasoning behind the choice of inocula and detection methodology is given in Sections 5.0 and 10.0.

Table 4-2 Types of inocula and detection methodology used in the semi-continuous system

Species		Inoculum type	Detection method
Common indicator pathogens			
	Escherichia coli	Bacterial cultures	Cultural
	Salmonella Typhimurium	Bacterial cultures	Cultural
Plant seeds			
Black grass	*Alopecurus myosuroides*	Seed	A germination test and tetrazolium stain test; (Noble *et al.,* 2011)
Tomato	*Lycopersicon esculentum*	Seed	A germination test and tetrazolium stain test; (Noble *et al.,* 2011)
Potato diseases			
Common scab	*Streptomyces scabies*	Infected tubers	Real-time PCR, based on thaxtomin toxin gene (*txtA)* (APHA in house test, already validated for DNA)
Powdery scab	*Spongospora subterranea*	Infected tubers	Plant bait test and Taqman PCR (Carnegie *et al*., 2009)
Late blight	*Phytophthora infestans*	Inoculated potato slices	Cultural; QRT-PCR: Llorente *et al*., 2010
Blackscurf	*Rhizoctonia solani*	Naturally infected tubers	Real-time PCR: Lees *et al*., 2002

	Species	Inoculum type	Detection method
Brown rot	*Ralstonia solanacearum*	Artificially infected plants of potato, tomato and/or eggplant	Real-time PCR test based on 16S rDNA (Weller *et al.,* 2000) and selective medium (SMSA)
Ring rot	*Clavibacter michiganensis* subsp. *sepedonicus*	Artificially infected plants of potato, tomato and/or eggplant	QRT-PCR from previous compost project combined with enrichment
Other crop diseases			
Basal rot of onion	*Fusarium oxysporum f.sp. cepae*	Cultured inoculum	Cultural: selective medium
Tomato foot and root rot	*F. oxysporum f.sp. radicis-lycopersici*	Cultured inoculum	Cultural: selective medium
Clubroot	*Plasmodiophora brassicae*	Naturally infected brassicas with club root (non-culturable)	Plant bait test and Taqman PCR (Noble *et al.,* 2011)

5.0 Results

5.1 *E. coli* & *Salmonella* (semi-continuous AD system)

The autoclaved feedstock was spiked with 9.3 x 10^6 cfu *E.coli* & *Salmonella* per ml, as measured by the Most Probable Number (MPN method), see Section 10.2.

For each treatment, results are presented which show the rate of die off for the microbial species, expressed as log concentration (MPN) per gramme of dry solids.

5.1.1 Pre-AD pasteurisation

Pasteurisation of the spiked feedstock at 70°C for one hour killed all *E. coli* and *Salmonella* in the spike.

5.1.2 Post-AD pasteurisation

E. coli and *Salmonella* were not detectable in any samples following (MAD) digestion and subsequent pasteurisation at 70°C for one hour.

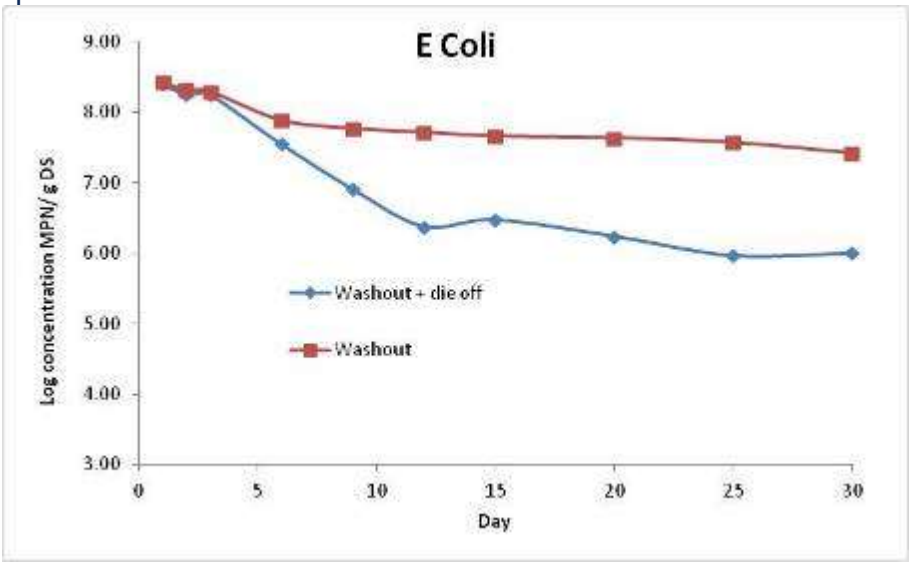
5.1.3 Mesophilic anaerobic digestion – no pasteurisation

After a 30 day period of MAD at 37.5°C, *E. coli* populations had declined by around 1.8-log, whilst *Salmonella* populations had declined by around 3.5-log.

Table 5-1 *E. coli* and *Salmonella* remaining in the reactor after 15 and 30 days (1 and 2 HRT), (log MPN / g DS)

Day	If washout alone occurring	*E. coli* (washout + die off)	*Salmonella* (washout + die off)
1	8.43	8.42	8.42
15	7.66	6.48	6.58
30	7.42	6.00	4.38

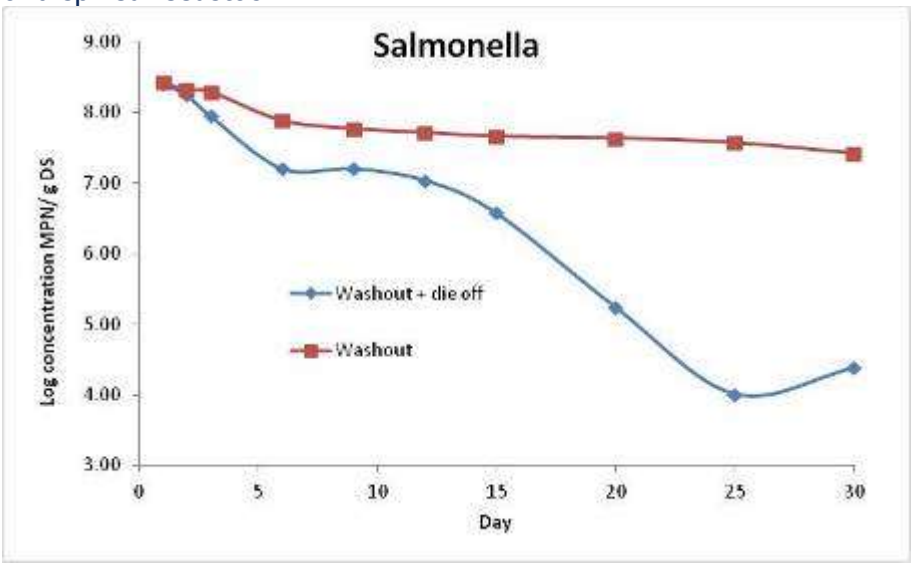
Figure 5-1 The number of E coli remaining in the MAD reactor following the addition of a spiked feedstock



The difference between the number of *E. coli* removed by wash-out (red line in Figure 5-1) and the numbers measured in the digestate (blue line) represents the fraction removed as a result of die-off in the reactor.

The reactor demonstrated almost perfect complete mixing, and the fraction of *E. coli* lost due to dilution follows a typical exponential dilution relationship (Figure 5-1). The difference between the two curves represents the die off due to the digestion process and this showed a 1.8 log removal. Gantzer *et al.* (2001) reported at best a two-log removal of faecal indicators during full-scale operation at retention times <26 days and UKWIR (1999) also reported a two-log *E. coli* removal. It is recognised that in practice a full-scale digester would be continuously fed with a feedstock containing *E. coli* and so the number of bacteria detected in the digestate would be much higher.

Figure 5-2 The number of Salmonella remaining in the MAD reactor following the addition of a spiked feedstock

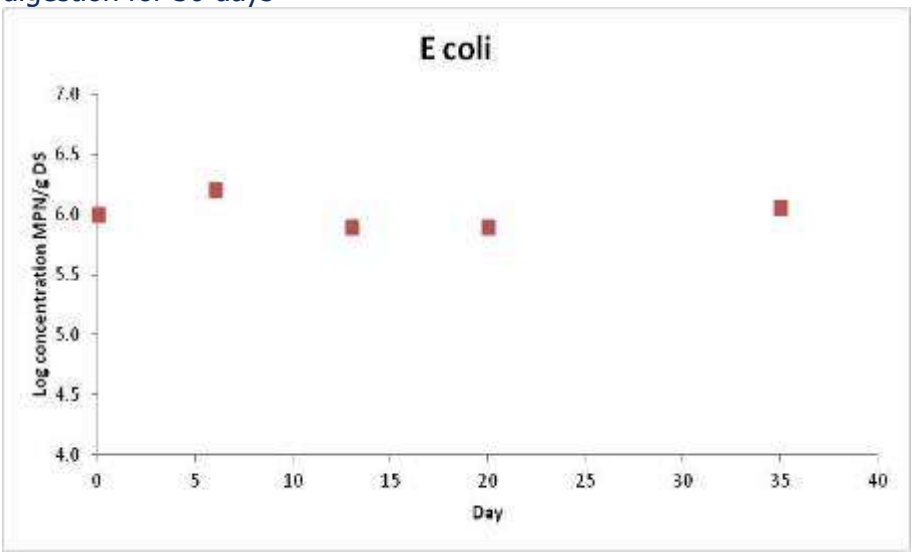


The difference between the number of *Salmonella* removed by wash-out (red line in Figure 5-2) and the numbers measured in the digestate (blue line) represents the fraction removed

as a result of die-off in the reactor. The estimated die-off was about 3.5log. Reports from the literature are of around a three-log removal of Salmonellae (Gantzer *et al.,* 2001; Guzmán *et al.*, 2007) during full-scale operation at retention times <26 days.

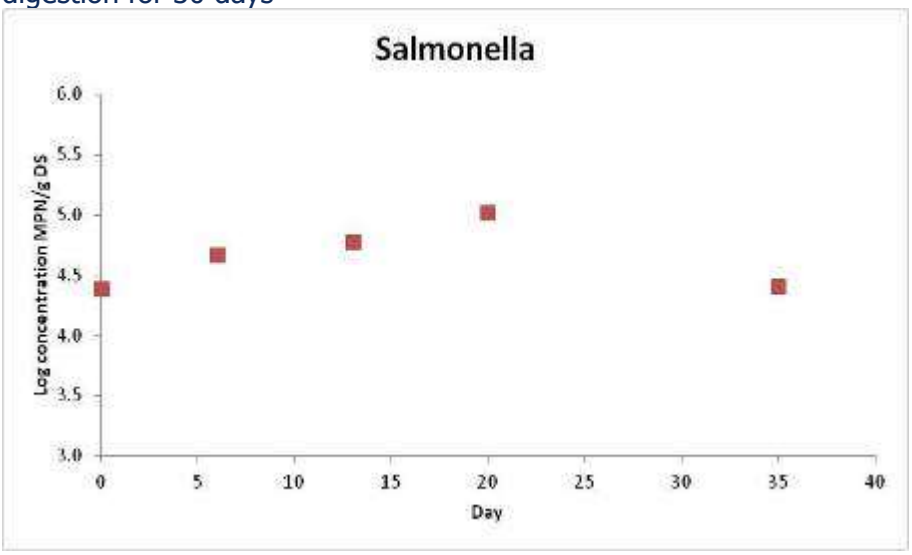
5.1.4 Storage of the digestate produced from MAD of the non-pasteurised feedstock

Figure 5-3 Survival characteristics of E. coli during digestate storage at 5°C following (MAD) digestion for 30 days



E. coli in the digestate showed good survival properties during storage at 5°C, and numbers were unchanged even after 35 days. By contrast *Salmonella* appeared to grow for a period of 20 days during storage at 5°C with numbers increasing from 4.4 to 5.0 log, after which a slow die-off occurred. Kearney *et al.* (1993a, b) found that the decline in viable numbers of *Salmonella* Typhimurium was temperature-dependent and was more rapid at 17°C than at 4°C in beef cattle slurry. However, the authors did not report growth.

Figure 5-4 Survival characteristics of Salmonella during digestate storage at 5°C following digestion for 30 days



5.2 Tomato and black grass seeds

5.2.1 Batch AD system

Seeds of the tomato cultivar Ailsa Craig (Mr Fothergill Seeds) and a natural source of mature black grass (*Alopecurus myosuroides*) (Pershore, Worcestershire) were used. Each sample bag contained 15 tomato seeds and 15 black grass seeds. Soft or atypical seeds were excluded.

To determine seed viability before and after the AD tests, a tetrazolium stain test was conducted on a sub-sample of seeds (Anon., 2006; Pollmann and Steiner, 1994). This required the seeds to be punctured with a needle and then immersed in P-buffered 1 % tetrazolium chloride solution (pH 7) at 30°C for 24 h. Seeds were then cut longitudinally through the midsection to expose both the embryo and endosperm. Staining of firm seeds was then assessed as red (viable), pink or white (non-viable). Soft, decaying seeds were also considered to be non-viable. All the six batches of untreated control tomato and black grass seeds contained at least 14 out of 15 viable seeds when tested in this way.

5.2.1.1 Pre- and post-AD pasteurisation

Pasteurisation at 70°C for one hour killed all tomato and black grass seeds (Table 5-2 and Table 5-3).

5.2.1.2 Mesophilic anaerobic digestion – no pasteurisation

Some tomato seeds were still viable after six days of MAD at 37.5°C (Table 5-2).
MAD at 37.5°C killed all black grass seeds after five days (Table 5-3).

5.2.2 Storage of the digestate produced from MAD of the non-pasteurised feedstock

Where not subjected to pasteurisation or digestion, tomato and black grass seeds were still viable following storage at between 7 and 11°C for ten days (Table 5-2 and Table 5-3).

Table 5-2 Effect of AD temperature and exposure time on tomato (cv. Ailsa Craig) seed viability

Treatment	Temp.	Time									
	ºC	1h		1d		5d		6d		10d	
		rep1	rep2	rep1	rep2	rep1	rep2	rep1	rep2	rep1	rep2
Pasteurisation	70	0	0	-	-	-	-	-	-	-	-
MAD	37.5	-	-	15	15	10	8	9	0	-	-
Storage	7-11	-	-	-	-	15	-	14	-	15	-

- not tested

Table 5-3 Effect of AD temperature and exposure time on black grass seed viability

Treatment	Temp.	Time									
	ºC	1h		1d		5d		6d		10d	
		rep1	rep2	rep1	rep2	rep1	rep2	rep1	rep2	rep1	rep2
Pasteurisation	70	0	0	-	-	-	-	-	-	-	-
MAD	37.5	-	-	10	13	0	0	0	0	-	-
Storage	7-11	-	-	-	-	14	-	14	-	13	-

- not tested

5.2.3 Semi-continuous AD system

Seeds of the tomato cultivar Ailsa Craig (Mr Fothergill Seeds) were used. 250 seeds were added to each reactor. To determine viability, seeds were sown into peat-based compost in seed trays (200 by 100 mm). Signs of germination were monitored determined daily.

5.2.3.1 Pre- and post-AD pasteurisation

Pasteurisation at 70°C for one hour killed all tomato seeds.

5.2.3.2 Mesophilic anaerobic digestion – no pasteurisation

MAD at 37.5°C killed all tomato seeds after three days.

The viability of tomato seeds that were placed in 40 mL digestate then immediately planted in compost was 100% (50 seeds germinated out of 50) (t0 samples). When attempts were made to recover seeds from digestate after 1 to 30 days after inoculation, only one viable seed was recovered two days after introduction (Table 5-4).

Table 5-4 Effect of AD exposure time on tomato (cv. Ailsa Craig) seed viability (number of remaining viable seeds from an estimated 20 seeds)

Treatment	Temp.	Time									
	ºC	1d		2d		3d		6d		9d	
		rep1	rep2	rep1	rep2	rep1	rep2	rep1	rep2	rep1	rep2
Pasteurisation	70	0	0	0	0	0	0	0	0	0	0
MAD	37.5	0	0	0	1	0	0	0	0	0	0

5.3 *Spongospora subterranea* (semi-continuous AD system)

Infected potato tubers with powdery scabs were used to harvest the cystosori (sporeballs) of *Spongospora subterranea*. A new scalpel blade was used to scrape cystosori and potato periderm from powdery scabs into a new, clean, 20 mL universal container. 0.74 g of this material was added to the inoculum mixture.

Following treatment, survival of pathogen was tested using tomato bait plants that had been grown in potting compost amended with digestate from non-pasteurised (Reactor 5 and 6) and pre-pasteurised (Reactors 7 and 8) treatments. Material from the bait plants themselves was then tested using PCR to confirm the presence of the organism.

5.3.1 Pre-AD pasteurisation

Spongospora subterranea could be detected in bait plants after pasteurisation at 70°C for one hour but could no longer be detected after three subsequent days of MAD at 37.5°C (Table 5-5).

5.3.2 Mesophilic anaerobic digestion – no pasteurisation

Spongospora subterranea could be detected in bait plants after twelve days (in one reactor only) following MAD at 37.5°C, but could no longer be detected after 15 days (Table 5-5).

5.3.3 Post-AD pasteurisation

Spongospora subterranea was not detectable following thirty days of MAD at 37.5°C digestion and subsequent pasteurisation at 70°C for one hour (Table 5-5).

Table 5-5 Effect of treatments on the level of *Spongospora subterranea* detected on tomato bait plants that had been growing in growing media with digestate added. DNA from bait plant roots were analysed by PCR for levels of pathogen

	Pre-pasteurisation		**No pasteurisation**		**Post-pasteurised**	
	Reactor 7	Reactor 8	Reactor 5	Reactor 6	Reactor 5	Reactor 6
Before spiking[1]	-ve	-ve	-ve	-ve		
After spiking	+ve	+ve	+ve	+ve		
After pasteurisation	+ve	+ve	-	-		
During digestion; 1 days after inoculation	+ve	+ve	+ve	-ve		
During digestion; 2 days after inoculation	-ve	+ve	-ve	-ve		
During digestion; 3 days after inoculation	-ve	-ve	-ve	-ve		
During digestion; 9 days after inoculation	-ve	-ve	-ve	-ve		
During digestion; 12 days after inoculation	-ve	-ve	+ve	-ve		
During digestion; 15 days after inoculation	-ve	-ve	-ve	-ve		
End of digestion; 30 days after inoculation	-ve	-ve	-ve	-ve		
After pasteurisation	-ve	-ve	-	-	-ve	-ve
During storage	-ve	-ve	-ve	-ve	-ve	-ve
End of storage	-ve	-ve	-ve	-ve	-ve	-ve

[1] On day 0, samples were collected from feedstock, pasteurised and non-pasteurised digestate prior to inoculating with pathogens. All initial samples prior to inoculation were negative.

5.4 Rhizoctonia solani AG3 (semi-continuous AD system)

Sclerotia of *Rhizoctonia solani* anastomosis group 3 (AG3) were prepared by growing PDA cultures (isolate Rs08) in 90 mm Petri dishes in the dark at 21°C. After 8 weeks, sclerotia were removed using a scalpel and air-dried overnight. Sclerotia were then macerated using a scalpel. Approximately 700 individual sclerotial fragments in 1 g of dried material with an average diameter of 2.16 mm (min 1.2 mm, max 3.45 mm) were added to the inoculation mixture.

Inoculum of the *R. solani* AG3 isolate tested positive by PCR prior to AD tests being conducted. Results of the TaqMan® PCR detection on samples exposed to AD tests are shown in Table 5-6. No RNA was detected in Reactors 5 and 6 beyond day 1 (RNA extracts had been depleted before these could be tested) except for one replicate PCR reaction from the Reactor 6 sample taken 6 and 15 days after inoculation. RNA from Reactors 7 and 8 (spiked feed stock pasteurised at 70°C for one hour) was detected up to 15 days after inoculation.

5.4.1 Pre-AD pasteurisation

Using RNA detection methods, *Rhizoctonia solani* could be detected after pasteurisation at 70°C for one hour (Table 5-6).

5.4.2 Mesophilic anaerobic digestion – no pasteurisation

Rhizoctonia solani was not detectable after fifteen days of MAD at 37.5°C (Table 5-6).

5.4.3 Post-AD pasteurisation

Rhizoctonia solani was not detectable after MAD at 37.5°C for thirty days and subsequent pasteurisation at 70°C (Table 5-6).

Table 5-6 Effect of AD treatments on the level of detectable of *Rhizoctonia solani* AG3 detected by reverse transcriptase nucleic acid (RNA) real-time PCR

	Pre-pasteurisation		**No pasteurisation**		**Post-pasteurised**	
	Reactor 7	Reactor 8	Reactor 5	Reactor 6	Reactor 5	Reactor 6
Before spiking	-ve	-ve	-ve	-ve		
After spiking	+ve	+ve	+ve	+ve		
After pasteurisation	+ve	+ve	-	-		
During digestion; 1 days after inoculation	+ve	+ve[a]	+ve[a]	+ve[a]		
During digestion; 2 days after inoculation	+ve	+ve	-ve	-ve		
During digestion; 3 days after inoculation	+ve	+ve	-ve	-ve		
During digestion; 6 days after inoculation	-ve	+ve	-ve	+ve		
During digestion; 9 days after inoculation	-ve	+ve	-ve	-ve		
During digestion; 12 days after inoculation	+ve	+ve	-ve	-ve		
During digestion; 15 days after inoculation	+ve	+ve	-ve	+ve		
During digestion; 20 days after inoculation	-ve	-ve	-ve	-ve		

	Pre-pasteurisation		No pasteurisation		Post-pasteurised	
	Reactor 7	Reactor 8	Reactor 5	Reactor 6	Reactor 5	Reactor 6
End of digestion; 30 days after inoculation	-ve	-ve	-ve	-ve		
After pasteurisation	-ve	-ve	-	-	-ve	-ve
During storage	-ve	-ve	-ve	-ve	-ve	-ve
End of storage	-ve	-ve	-ve	-ve	-ve	-ve

[a] Detected by DNA PCR only

Detection of nucleic acids alone does not mean that the pathogen is alive, although lack of detection suggests it is no longer viable. Nucleic acid may be surviving inside large clumps of sclerotia. Therefore it is difficult to be sure that pasteurisation is improving kill before or after MAD.

5.5 *Phytophthora* species (batch AD system)

Inocula of *Phytophthora cinnamomi* and *P. nicotianae* isolates were prepared in a peat-based medium. Lime (Dolokal, 8 g l^{-1}), fertiliser (PG mix 15N-10P-20K, 0.8 g l^{-1}) and oat flakes (40 g l^{-1}) were added to dry peat. The medium was moistened to 50 % w/w moisture, sterilised and inoculated with the *Phytophthora* cultures grown on potato dextrose agar (PDA). The peat-based media were incubated at 20°C for 21 days, after which they contained hyphae and hyphal swellings. Sample bags contained 3 g peat inoculum. After exposure to the various treatments, a serial dilution of the material from each of the retrieved bags was conducted on three replicate plates using PDA + PARPH agar (Mitchell *et al*., 1986). A sample of 1.3 g was used to allow for the increase in fresh weight of the original 1 g samples in the digestate. The plates were recorded for *Phytophthora* after three days incubation at 20°C by counting colony forming units (cfu) with mycelium, hyphal swellings, and sporangia typical of *Phytophthora*. The detection limit of the test was 34 cfu g^{-1} peat-based medium.

Phytophthora infestans inoculum was produced on PDA. Plugs of inoculum were then placed on potato slices and incubated at 20°C for 5 days. Cubes (25 mm sides) of infected potato tissue were then used in the AD tests. Viability of *P. infestans* on retrieved cubes was tested by placing them on fresh potato slices in a plastic box at 20°C for 5 days and then examining the potato slices for sporangia typical of *P. infestans*.

5.5.1 Pre- and post-AD pasteurisation

After pasteurisation at 70°C *Phytophthora cinnamomi*, *Phytophthora nicotianae* and *Phytophthora infestans* could not be detected (Table 5-7, Table 5-8 and Table 5-9).

5.5.2 Mesophilic anaerobic digestion – no pasteurisation

After MAD at 37.5°C for one day *Phytophthora cinnamomi*, *Phytophthora nicotianae* and *Phytophthora infestans* could not be detected (Table 5-7, Table 5-8 and Table 5-9).

5.5.3 Storage of the digestate produced from MAD of the non-pasteurised feedstock

Having been spiked into digestate stored at between 7 and 11°C *Phytophthora cinnamomi* and *Phytophthora nicotianae* could not be detected after five days (Table 5-7 and Table 5-8). *Phytophthora infestans* could not be detected after one day (Table 5-9).

Table 5-7 Effect of AD temperature and exposure time on *Phytophthora cinnamomi* viability (cfu/g peat detected) in two replicates. Initial peat inoculum contained 9.5 x 10^3 to 6.7 x 10^4 cfu/g

Treatment	Temp.	Time					
	ºC	1h		1d		5d	
		rep 1	rep 2	rep 1	rep 2	rep 1	rep 2
Pasteurisation	70	0	0	-	-	-	-
MAD	37.5	1.0 x 10^4	1.2 x 10^4	0	0	0	-
Storage	7-11	8.7 x 10^3	4.0 x 10^4	3.0 x 10^2	2.7 x 10^3	0	0

- not tested

Table 5-8 Effect of AD temperature and exposure time on *Phytophthora nicotianae* viability (cfu/g peat detected) in two replicates. Initial peat inoculum contained 5.3 x 10^3 to 4.0 x 10^4 cfu/g

Treatment	Temp.	Time					
	ºC	1h		1d		5d	
		rep 1	rep 2	rep 1	rep 2	rep 1	rep 2
Pasteurisation	70	0	0	-	-	-	-
MAD	37.5	2.3 x 10^3	1.2 x 10^4	0	0	0	-
Storage	7-11	2.7 x 10^3	1.2 x 10^4	1.0 x 10^3	3.0 x 10^3	0	0

- not tested

Table 5-9 Effect of AD temperature and exposure time on *Phytophthora infestans* viability in two replicates

Treatment	Temp.	Time					
	ºC	1h		1d		5d	
		rep 1	rep 2	rep 1	rep 2	rep 1	rep 2
Pasteurisation	70	o	o	-	-	-	-
MAD	37.5	+	o	o	o	o	-
Storage	7-11	+	+	o	o	o	-

+ detected o not detected - not tested

5.6 Fusarium species

5.6.1 Fusarium culmorum and *F oxysporum formae specialis radicis-lycopersici* (batch AD system)

Petri dish cultures of *F. culmorum* and *F. oxysporum f.sp. radicis-lycopersici* were grown on PDA which, when flooded with 20 ml sterile distilled water, were used to produce mycelial and conidial suspensions for inoculating 66 g sterilised talc. The talc was kept for 6 weeks at 20°C to allow chlamydospores to develop. Sample bags contained 1.25 g talc inoculum. After exposure to the various treatments, 1.3 g wet talc (equivalent to 1 g dry original talc) was obtained from the retrieved bags and added to 9 ml of sterile distilled water. The suspension (0.1 ml) was plated on to each of three replicate plates of PDA + chlorotetracycline which were then incubated at 22°C for 6 days. Viable chlamydospores were detected by counting cfu with mycelium and conidia typical of *F. culmorum* and *F. oxysporum* with a similar detection threshold to the *Phytophthora* method (34 cfu g^{-1}).

5.6.1.1 Pre- and post-AD pasteurisation

Fusarium culmorum and *Fusarium oxysporum f.sp radicis-lycopersici* could not be detected after pasteurisation at 70°C for one hour (Tables 5-10 and 5-11).

5.6.1.2 Mesophilic anaerobic digestion – no pasteurisation

Fusarium culmorum and *Fusarium oxysporum f.sp radicis-lycopersici* could not be detected after MAD at 37.5°C for one day (Tables 5-10 and 5-11).

5.6.1.3 Storage of the digestate produced from MAD of the non-pasteurised feedstock

When spiked into digestate stored at between 7 and 11°C, *Fusarium culmorum* and *Fusarium oxysporum f.sp radicis-lycopersici* could not be detected after five days (Tables 5-10 and 5-11).

Table 5-10 Effect of AD temperature and exposure time on *Fusarium culmorum* viability (cfu/g talc detected) in two replicates. Initial talc inoculum contained 5.6 x 10^5 to 6.3 x 10^5cfu/g

Treatment	Temp.	Time					
	ºC	1h		1d		5d	
		rep 1	rep 2	rep 1	rep 2	rep 1	rep 2
Pasteurisation	70	0	0	-	-	-	-
MAD	37.5	1.4 x 10^5	0	0	0	0	-
Storage	7-11	1.2 x 10^4	5.3 x 10^5	4.3 x 10^4	3.0 x 10^4	0	0

not tested

Table 5-11 Effect of AD temperature and exposure time on *Fusarium oxysporum f.sp radicis-lycopersici* viability (cfu/g talc detected) in two replicates. Initial talc inoculum contained 1.5 x 10^6 to 2.3 x 10^6cfu/g

Treatment	Temp.	Time					
	ºC	1h		1d		5d	
		rep 1	rep 2	rep 1	rep 2	rep 1	rep 2
Pasteurisation	70	0	0	-	-	-	-
MAD	37.5	1.0 x 10^5	7.7 x 10^4	0	0	0	-
Storage	7-11	2.7 x 10^4	1.5 x 10^6	4.0 x 10^4	1.3 x 10^4	0	0

- not tested

5.6.2 Fusarium culmorum (semi-continuous AD system)

An isolate of *Fusarium culmorum* (FC1868, isolated in 2011 from an ear of winter wheat from Leicestershire) was used in this study. Plugs were removed from the culture collection in APHA, placed onto Sucrose Nutrient Agar (SNA) and incubated at 18°C under UV light (12 hours/12 hours) for 7 days. To bulk-up the inoculum a spore suspension was created by flooding the plate with 5mL of sterile distilled water and gently removing the spores using a sterile hockey stick spreader. 100 µL of spore suspension was then used to inoculate further SNA plates which were incubated under the same conditions as described previously before the spores (macroconidia) were removed. Cultures were grown for approximately four weeks until sufficient inoculum was available.

Inoculum of the *F. culmorum* isolate tested positive by PCR prior to AD tests being conducted. Feedstock and anaerobic digestate prior to spiking were negative for *F. culmorum* DNA. Results of the TaqMan® PCR detection on samples exposed to AD tests are shown in Table 5-12.

5.6.2.1 Pre-AD pasteurisation

Using nucleic acid detection methods, *Fusarium culmorum* could be detected after pasteurisation at 70°C for one hour (Table 5-12).

5.6.2.2 Mesophilic anaerobic digestion – no pasteurisation

F. culmorum was not detectable following a three day period of MAD at 37.5°C (Table 5-12).

5.6.2.3 Mesophilic anaerobic digestion (post-MAD pasteurised)

Fusarium culmorum was not detectable after 30 days of MAD and pasteurisation at 70°C (Table 5-12).

Table 5-12 Effect of AD exposure time on the level of *Fusarium culmorum* detected by reverse transcriptase nucleic acid (RNA) PCR

	Pre-pasteurisation		No pasteurisation		Post-pasteurised	
	Reactor 7	Reactor 8	Reactor 5	Reactor 6	Reactor 5	Reactor 6
Before spiking	-ve	-ve	-ve	-ve		
After spiking	+ve	+ve	+ve	+ve		
After pasteurisation	+ve	+ve	-	-		
During digestion; 1 days after inoculation	+ve	+ve	-ve	+ve		
During digestion; 2 days after inoculation	+ve	+ve	+ve	-ve		
During digestion; 3 days after inoculation	-ve	+ve	-ve	+ve		
During digestion; 6 to 20 days after inoculation	-ve	-ve	-ve	-ve		
End of digestion; 30 days after inoculation	-ve	-ve	-ve	-ve		
After pasteurisation	-ve	-ve	-	-	-ve	-ve
During storage	-ve	-ve	-ve	-ve	-ve	-ve
End of storage	-ve	-ve	-ve	-ve	-ve	-ve

Shorter periods at equivalent temperatures were required in the batch system than in the semi-continuous AD system before *Fusarium culmorum* inoculum was reduced to below a detectable level. This may have been due to the detection method used following the batch system (mycelial growth on selective agar plates) whereas detection based on nucleic acids may have detected un-degraded but non-viable inoculum (from the semi-continuous system).

5.7 *Plasmodiophora brassicae* (batch AD system)

Two sources of infected 'galled' cabbage root with adhering soil were used as inoculum in the tests. Sample bags contained 3 g of galls and adhering soil. After AD, the gall material retrieved from the bags was tested for *P. brassicae* presence using a TaqMan® PCR detection method (Noble *et al.* 2011) and clubroot bioassay modified from Fayolle *et al.* (2006).

Gall inoculum of each sample was mixed with peat-based growing medium and filled into 300 ml pots. A Chinese cabbage seedling (cv. Rocco) was potted in each pot and grown for six weeks, after which the plant was uprooted and assessed for clubroot symptoms expression on a 0–3 scale (Fayolle *et al.,* 2006). Plants (3) were also potted in growing media containing non-digested gall inoculum of each isolate (positive controls) or without gall inoculum (negative controls).

The TaqMan® PCR detection method was repeated on all root material and adhering growing medium of bioassay plants grown with the above samples. Results from the TaqMan® PCR were categorised as positive (CT <32), weak positive (CT > 32), and negative.

Before AD tests were conducted, root and soil inoculum of both *P. brassicae* isolates tested positive using the TaqMan® PCR detection method and the Chinese cabbage clubroot bioassay. The negative control test plants tested negative with both Chinese cabbage plant bioassay and the TaqMan® PCR test.

5.7.1 Pre- and post-MAD pasteurisation

Based on Chinese cabbage clubroot bioassays and TaqMan® PCR tests on the bioassay plant material, inoculum of two *Plasmodiophora brassicae* isolates could not be detected after pasteurisation at 70°C for one hour (Table 5-13 and Table 5-14). Although both isolates were detected after one hour at 70°C from TaqMan® PCR tests, this may have been due to the presence of un-degraded, non-viable inoculum. After one day at 70°C, both *P. brassicae* isolates tested negative from the TaqMan® PCR tests alone (Table 5-13).

5.7.2 Mesophilic anaerobic digestion – no pasteurisation

Based on Chinese cabbage clubroot bioassays and TaqMan® PCR tests on the bioassay plant material, inoculum of two *Plasmodiophora brassicae* isolates could not be detected after exposure to MAD at 37.5°C for one day (Table 5-14 and Table 5-15). Although one of the isolates was detected after MAD at 37.5°C for 6 days from TaqMan® PCR tests, this may have been due to the presence of un-degraded, non-viable inoculum (Table 5-13).

5.7.3 Storage of the digestate produced from MAD of the non-pasteurised feedstock

Based on Chinese cabbage clubroot bioassays and TaqMan® PCR tests on the bioassay plant material, inoculum of two *Plasmodiophora brassicae* isolates could not be detected after being 'stored' at 7-11°C for six days (Table 5-14 and Table 5-15). One of the isolates was detected after storage at 7-11°C for ten days from TaqMan® PCR tests, this may have been due to the presence of un-degraded, non-viable inoculum (Table 5-13).

Table 5-13 Effect of AD temperature and exposure time on TaqMan® PCR test results of inoculum of two *Plasmodiophora brassicae* isolates in two replicates, before plant bioassay

Treatment	Temp	Isolate	Time									
	ºC		1h		1d		5d		6d		10d	
			rep 1	rep 2	rep 1	rep 2	rep 1	rep 2	rep 1	rep 2	rep 1	rep 2
Pasteurisation	70	Welles.	++	+	o	-	-	-	-	-	-	-
		Warw.	++	-	o	-	-	-	-	-	-	-
MAD	37.5	Welles.	-	-	++	+	+	+	++	-	-	-
		Warw.	-	-	-	-	-	-	-	-	-	-
Storage	7-11	Welles.	++	-	++	-	++	-	++	-	++	-
		Warw.	++	-	-	-	++	-	-	-	-	-

++ positive + weak positive o negative - not tested

Table 5-14 Effect of AD temperature and exposure time on Chinese cabbage clubroot bioassay results of inoculum of two *Plasmodiophora brassicae* isolates in two replicates

Treatment	Temp	Isolate	Time									
	ºC		1h		1d		5d		6d		10d	
			rep 1	rep 2	rep 1	rep 2	rep 1	rep 2	rep 1	rep 2	rep 1	rep 2
Pasteurisation	70	Welles.	o	o	-	-	-	-	-	-	-	-
		Warw.	o	-	-	-	-	-	-	-	-	-
MAD	37.5	Welles.	-	-	o	o	o	o	o	-	-	-
		Warw.	-	-	o	-	-	-	-	-	-	-
Storage	7-11	Welles.	++	-	o	-	o	-	o	-	-	-
		Warw.	++	-	-	-	o	-	o	-	-	-

++ positive + weak positive o negative - not tested

Table 5-15 TaqMan® PCR test results of bioassay plant roots grown in contact with inoculum of two *Plasmodiophora brassicae* isolates exposed to different digestate time-temperature treatments in two replicates

Treatment	Temp	Isolate	Time									
	ºC		1h		1d		5d		6d		10d	
			rep 1	rep 2	rep 1	rep 2	rep 1	rep 2	rep 1	rep 2	rep 1	rep 2
Pasteurisation	70	Welles.	o	o	-	-	-	-	-	-	-	-
		Warw.	o	-	-	-	-	-	-	-	-	-
MAD	37.5	Welles.	-	-	o	o	o	o	o	-	-	-
		Warw.	-	-	o	-	-	-	-	-	-	-
Storage	7-11	Welles.	++	-	o	-	o	-	o	-	-	-
		Warw.	++	-	-	-	++	-	o	-	-	-

++ positive + weak positive o negative - not tested

5.8 *Clavibacter michiganensis* ssp *sepedonicus* and *Ralstonia solanacearum* (semi-continuous AD system

Ralstonia solanacearum isolate NCPPB 909 and *Clavibacter michigenensis* subsp. *sepedonicus* isolate NCPB 4053 were obtained from the National Collection of Plant Pathogenic Bacteria and cultured on sucrose peptone agar and nutrient dextrose agar respectively. Cultures from the agar plates were suspended in phosphate buffer and diluted to concentrations of $1.6x10^{10}$ (*Ralstonia*) and $6x10^{9}$ (*Clavibacter*) colony-forming units per ml. These suspensions were then used to inoculate the feedstock material.

5.8.1 Detection of bacteria using real time PCR

Initial testing by real time PCR showed that DNA of both *Ralstonia* and *Clavibacter* populations were readily detectable in samples of inoculated feedstock, both before and after pasteurisation at 70°C for 1 hour. Mean inoculum populations detected by real-time PCR in the reactors prior to digestion were equivalent to 4.6×10^{7} cells per ml of *R. solanacearum* and 4.9×10^{7} cells per ml of *C. michiganensis* ssp. *sepedonicus*. Neither pathogen was detected in un-inoculated feedstock.

Initial plans to estimate viability of the bacteria based on RNA detection failed since the PCR assays used were not suitable for conversion to reverse-transcriptase PCR. It was therefore not possible to directly assess the effect of pasteurisation on inoculum viability, although it is already widely accepted that these pathogens will be readily killed at this temperature-time combination.

So that pathogen viability could be subsequently investigated in digestate samples, an alternative bio-PCR method was developed based on quantifying any increase in target DNA from viable populations during enrichment in selective media. This was then employed from the 6^{th} day of anaerobic digestion to investigate the viability of the original inoculum.

The bio-PCR tests were performed before and after enrichment of the bacteria by adding 100 µL aliquots of digestate to 20 ml selective broth media and incubating for 24 hr. For *Ralstonia*, enrichment in SMSA broth (Elphinstone *et al.*, 1996) at 28°C was used, whereas *Clavibacter* was enriched in MNTA broth (Jansing and Rudolph, 1998; Schaad *et al.*, 1999) at 21°C. The broth cultures were sampled by removal of 100 µL before and after incubation. These aliquots were heated to 100°C for 5 minutes prior to using 1.0 µL in specific PCR reactions. Any increase in bacterial populations after enrichment was indicated by a subsequent decrease in the critical threshold (C_T) values of the real time PCR result, which was taken as an indication of viability.

5.8.2 Pre-AD pasteurisation + MAD

When feedstock was pasteurised prior to anaerobic digestion (Reactors 7 and 8) *Ralstonia* and *Clavibacter* DNA was less readily detected by the real-time PCR test as digestion time increased, falling near or below the detection limit around day 15. These results can be explained by the fact that pasteurisation will readily lyse the bacterial cells, thus exposing DNA to enzymatic degradation. In the absence of the pre-pasteurisation treatment, the DNA remains protected inside entire bacterial cells and less exposed to degradation. As with the non-pasteurised feedstocks, bio-PCR tests showed that all bacteria detected were non-viable from at least the 6^{th} day of anaerobic digestion.

5.8.3 MAD + post-AD pasteurisation

Since both pathogens were killed during the anaerobic digestion process, no further investigation of survival during post-pasteurisation or storage was conducted.

5.8.4 Mesophilic anaerobic digestion (MAD) – no pasteurisation

Both *Clavibacter* and *Ralstonia* DNA remained readily detectable over the 30 day anaerobic digestion period when introduced in non-pasteurised feedstock. However, introduction of the bio-PCR test from the 6th day of anaerobic digestion showed that none of the bacteria were able to grow during enrichment and had therefore been rendered non-viable during the process (Table 5-16 and Table 5-17).

Table 5-16 Effect of treatments on detection of viable *Ralstonia solanacearum*

	Pre-pasteurised		No pre-pasteurisation	
	Reactor 5	Reactor 6	Reactor 7	Reactor 8
Before spiking	-ve	-ve	-ve	-ve
After spiking	ND	ND	ND	ND
After pasteurisation	ND	ND	ND	ND
During digestion; 1 to 3 days after inoculation	ND	ND	ND	ND
During digestion; 6 to 20 days after inoculation	-ve	-ve	-ve	-ve
End of digestion (30 days after inoculation)	-ve	-ve	-ve	-ve
After pasteurisation	NT	NT	NT	NT
During storage	NT	NT	NT	NT
End of storage	NT	NT	NT	NT

ND = Pathogen detected by real-time PCR but viability not determined by bio-PCR (bio-PCR testing only available from 6th day of digestion)
NT = Not tested
-ve = Viable populations not detected by bio-PCR

Table 5-17 Effect of treatments on detection of viable *Clavibacter michiganensis* subsp. *sepedonicus*

	Pre-pasteurised		No pre-pasteurisation	
	Reactor 5	Reactor 6	Reactor 7	Reactor 8
Before spiking	-ve	-ve	-ve	-ve
After spiking	ND	ND	ND	ND
After pasteurisation	ND	ND	ND	ND
During digestion; 1 to 3 days after inoculation	ND	ND	ND	ND
During digestion; 6 to 20 days after inoculation	-ve	-ve	-ve	-ve
End of digestion (30 days after inoculation)	-ve	-ve	-ve	-ve
After pasteurisation	NT	NT	NT	NT
During storage	NT	NT	NT	NT
End of storage	NT	NT	NT	NT

ND = Pathogen detected by real-time PCR but viability not determined by bio-PCR (bio-PCR testing only available from 6th day of digestion)
NT = Not tested
-ve = Viable populations not detected by bio-PCR.

5.9 Streptomyces scabies (semi-continuous AD system)

Infected tubers containing inoculum of the *S. scabies* isolate tested positive by PCR prior to AD tests being conducted. Results of the TaqMan® PCR detection on samples exposed to AD tests are shown in Table 5-18. DNA was detected in digestate up to nine days after inoculation in Reactors 5 and 6 (no pasteurisation) and Reactors 7 and 8 (pasteurised). It was not possible to produce an RNA assay because the DNA assay does not have an RNA equivalent. Therefore the results are likely to overestimate survival particularly where the digestate has been heat treated.

5.9.1 Pre-AD pasteurisation + MAD

Using DNA detection methods, *S. scabies* could be detected after pasteurisation (Table 5-18).

5.9.2 MAD + post-AD pasteurisation

Streptomyces scabies was not detectable using DNA detection methods after thirty days of MAD followed by pasteurisation at 70°C for one hour (Table 5-18).

5.9.3 Mesophilic anaerobic digestion (MAD) – no pasteurisation

Streptomyces scabies was not detectable following a nine day period of MAD at 37.5°C (Table 5-18).

Table 5-18 Effect of treatments on *Streptomyces scabies*. Using DNA test

	Pre-pasteurisation		No pasteurisation		Post-pasteurised	
	Reactor 7	Reactor 8	Reactor 5	Reactor 6	Reactor 5	Reactor 6
Before spiking	-ve	-ve	-ve	-ve		
After spiking	+ve	+ve	+ve	+ve		
After pasteurisation	+ve	+ve	-	-		
During digestion; 1 days after inoculation	+ve	+ve	+ve	-ve		
During digestion; 2 days after inoculation	+ve	-ve	+ve	+ve		
During digestion; 3 days after inoculation	+ve	+ve	+ve	+ve		
During digestion; 6 days after inoculation	-ve	+ve	+ve	+ve		
During digestion; 9 days after inoculation	+ve	-ve	+ve	-ve		
During digestion; 12 days after inoculation	-ve	-ve	-ve	-ve		
During digestion; 15 days after inoculation	-ve	-ve	-ve	-ve		
During digestion; 20 days after inoculation	-ve	-ve	-ve	-ve		
End of digestion (30 days after inoculation)	-ve	-ve	-ve	-ve		
After pasteurisation	-ve	-ve	-	-	-ve	-ve
During storage	-ve	-ve	-ve	-ve	-ve	-ve
End of storage	-ve	-ve	-ve	-ve	-ve	-ve

6.0 Summary of the effect of anaerobic digestion processes on pathogens and seeds used in the experimental investigations

6.1 Plasmodiophoromyces

Two members of the Plasmodiophoraceae were used in the experimental investigations: *Plasmodiophora brassicae* (clubroot of brassicas) and *Spongospora subterranea* (powdery scab of potato). Both of these are common pathogens of agricultural crops in the UK and would be able to infect crop plants if they survived the AD process and the digestate was applied to agricultural land.

A review of the literature (Section 8.2.1) identified several reports of clubroot surviving the AD process, for example for up to 14 days at 55°C, but no literature data on *S. subterranea* were found. A related organism, *Polymyxa betae,* was reported as being reduced to below detectable levels in AD after 4 days at 55°C.

P. brassicae and *S. subterranea* inocula were introduced into model AD systems. The presence of both organisms was determined using a methodology which included a plant bait test and subsequent PCR test on the bait plants.

6.1.1 Plasmodophora brassicae

Clubroot was reduced to below detectable levels after pasteurisation for one hour at 70°C, after one day at 37.5°C in MAD, or after six days when spiked into digestate stored at 7-11°C.

6.1.2 Spongospora subterranea

Spongospora was still detectable after pasteurisation at 70°C for one hour – although was not detectable when pasteurised material had been subjected to MAD at 37.5°C for three days. The organism was not detectable in digestate subjected to MAD at 37.5°C for twelve days (with no pre-pasteurisation). It was not spiked into stored digestate.

6.2 Fungi

A range of common fungal pathogens of plants were used to test survival through the AD processes. These included *Phytophthora infestans* (late blight of potato)*, P. cinnamomi, P. nicotianae*, *Fusarium culmorum, F. oxysporum, F. radicis lycopersicae* and *Rhizoctonia solani*.

6.2.1 Phytophthora spp

In experimental investigations *Phytophthora infestans, Phytophthora cinnamomi* and *P. nicotianae* could not be detected after pasteurisation for one hour at 70°C.

Phytophthora infestans, Phytophthora cinnamomi and *P. nicotianae* could not be detected after one day at 37.5°C in MAD

Phytophthora cinnamomi and *P. nicotianae* could not be detected 5 days after spiking into digestate storage in digestate at 7-11°C.

P. infestans could not be detected one day after spiking into digestate stored at 7-11°C.

6.2.2 Fusarium spp

In batch AD experiments, *Fusarium culmorum* and *Fusarium oxysporum f.sp radicis-lycopersici* could not be detected after pasteurisation for one hour at 70°C or one day at 37.5°C in MAD (with no pre-pasteurisation) or five days after being spiked into digestate stored at 7-11°C.

F. culmorum was also tested in the semi-continuous AD system. Using DNA PCR detection methods, it could be detected after pasteurisation at 70°C for one hour. It was also detectable at up to three days in MAD at 37.5°C (no pre-pasteurisation). However, RNA PCR tests could not detect *F.culmorum* after three days in either treatment, suggesting that it was no longer viable.

6.2.3 Rhizoctonia solani

RNA of *R. solani* was detectable following pasteurisation at 70°C for one hour. It was still detectable at up to fifteen days following pasteurisation. Where pre-pasteurisation had not been applied, RNA was also detectable at up to fifteen days in MAD at 37.5°C using a PCR test.

6.3 Bacterial plant pathogens

6.3.1 Ralstonia solanacearum (brown rot of potato)

R. solanacearum is a quarantine disease in the UK and therefore not commonly found in UK potato feedstocks. There are some literature reports that *R. solanacearum* can survive up to 30 days at 38°C in MAD but that it is eliminated within a day in AD at 52°C.

Experimental investigations using semi-continuous AD and a bio-PCR detection approach suggested that brown rot was undetectable after six days MAD at 37.5°C. No data were collected to demonstrate specific impacts of pasteurisation on this organism.

6.3.2 Clavibacter michiganensis ssp. sepedonicus (ring rot of potato)

C. michiganensis ssp. *sepedonicus* is a quarantine disease in the UK and therefore not likely to be common in feedstocks. Reports from the literature have suggested that ring rot can survive up to six hours but not 24 hours in AD at 37°C and 1-6 months in stored digestate.

Experimental investigations using semi-continuous AD and a bio-PCR detection approach suggested that ring rot was undetectable after six days MAD at 37.5°C. No data were collected to demonstrate specific impacts of pasteurisation on this organism.

6.3.3 Streptomyces scabies (common scab)

S. scabies is common in soils in the UK and is therefore likely to be present in UK-sourced feedstocks.

Experimental investigations using semi-continuous AD suggested that *S. scabies* DNA was still detectable following pasteurisation at 70°C for one hour, but that it was not detectable following a subsequent 12 days of MAD at 37.5°C. Likewise, where pre-pasteurisation had not been applied, DNA was undetectable after 12 days of MAD at 37.5°C.

6.4 Seeds

Two types of seed were used in the experimental investigations: tomato seed (*Lycopersicum esculentum* cv. Ailsa Craig) and black grass seed (*Alopecurus myosuroides*). There was no information on survival of black grass seed in the literature and there were conflicting reports of survival of tomato seed.

Both types of seed survived well when spiked into digestate stored at 7-11°C, remaining viable at the end of the ten day test period. However, neither survived pasteurisation for one hour at 70°C. In batch tests with no pre-pasteurisation, tomato seed survived MAD at 37.5°C for up to six days and black grass seed survived MAD at 37.5°C for up to five days. In the semi-continuous system, tomato seeds were killed by pasteurisation at 70°C for one hour. With no pre-pasteurisation, tomato seed only survived at low levels for up to three days in MAD at 37.5°C within this system. (Black grass seed was not tested in the semi-continuous system).

6.5 Human bacterial pathogens

Experimental investigations using semi-continuous MAD at 37.5°C demonstrated a 1.8 log die-off for *Escherichia coli* and a c.3.5 log die-off for *Salmonella* spp. This broadly aligns with the reductions reported for MAD in the literature. Higher temperatures would be expected to accelerate this reduction, with digestate being effectively sterilised by pasteurisation at 70°C.

During storage at 5°C, *E. coli* numbers survived unchanged for up to 35 days, whereas *Salmonella* spp. grew for up to 20 days, increasing from 4.4 to 5.0 logs, before starting to decrease.

6.6 Summary

Table 6-1 and Table 6-2 provide a summary of the experimental results obtained. Overall, pasteurisation is, as expected, an effective treatment for reducing the survival of most pathogens and seeds tested. The most resilient organism was found to be *S. subterranea,* which is well known to survive for long periods as cystosori and to be difficult to eradicate. The similar organism, *P. brassicae,* appeared to be less resilient, although this is based on only a few positive results. Tomato seed also proved to be resilient in MAD, especially in the batch process.

The MAD process appeared to eliminate most organisms after 20 days but many survived at least five days. Some of the results were difficult to interpret, for example, results from tests for *S. scabies* and *R. solani* appear to suggest that these organisms can survive pasteurisation and long subsequent periods in MAD, but because the detection of these organisms was based solely on the presence of RNA or DNA it is impossible to say whether viable organisms survived the process and would therefore prove to be a risk to crops.

Some organisms survived storage at 7-11°C for up to 10 days, particularly seed.

Table 6-1 Time after which levels of inoculated organisms dropped below detection limits (LOD). Batch AD results

Organism	Pasteurisation 70°C	MAD 37.5°C	Stored in digestate at 7-11°C	Detection method(s)
Tomato seed (*Lycopersicum esculentum*) cv Ailsa Craig	1 hour	Still viable at 6 days	Still viable at 10 days	Tetrazolium staining
Black grass seed (*Alopecurus myosuroides*)	1 hour	5 days	Still viable at 10 days	Tetrazolium staining
Phytophthora infestans	1 hour	1 day	1 day	Culture
Phytophthora cinnamomi	1 hour	1 day	5 days	Culture
Phytophthora nicotianae	1 hour	1 day	5 days	Culture
Fusarium culmorum	1 hour	1 day	5 days	Culture
Fusarium oxysporum f. sp. radicis lycopersicae	1 hour	1 day	5 days	Culture
Plasmodiophora brassicae	1 hour	1 day	6 days	PCR, Bioassay, Bioassay+PCR

Table 6-2 Time after which levels of inoculated organisms dropped below detection limits (LOD). Semi-continuous AD results

Organism	MAD + Pre-Pasteurisation	MAD	Detection method
Escherichia coli	1 hour	1.8 log decrease at 30 days	Culture
Salmonella	1 hour	3.5 log decrease at 30 days	Culture
Tomato seed (*Lycopersicum esculentum*) cv Ailsa Craig	1 hour	3 days	Germination
Streptomyces scabies	12 days	12 days	DNA PCR
Spongospora subterranea	3 days	12 days	Bait test+DNA PCR
Rhizoctonia solani	15 days	15 days	DNA PCR RNA PCR
Ralstonia solanacearum	6 days	6 days	BIOPCR
Clavibacter michiganensis subsp. *sepedonicus*	6 days	6 days	BIOPCR
Fusarium culmorum	3 days	3 days	DNA PCR RNA PCR

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Appendix 1 Literature review: Fates of some common pests and diseases in AD

8.0 Overview

The literature review showed that specific knowledge of the survival of plant pathogens in AD processes is patchy and that it is difficult to generalise across types of pathogens and processes. Several fungal plant pathogens including different *Fusarium* species, *Rhizoctonia solani*, and *Sclerotium cepivorum* have been shown to be eradicated in AD at 40°C after 21 days or less. *Verticillium albo-atrum* and *V. dahlia* have been shown to be eradicated after 28 days in MAD at 38°C. These results, together with those for *P. brassicae*, indicate that some plant pathogens may be more temperature sensitive in AD than in composts (Noble *et al*. 2009, 2011), possibly due to the higher moisture content and presence of inhibitory substances produced in AD.

Seigner *et al.* (2010) showed that plant pathogen kill was improved in anaerobic digestate compared with the same time-temperature treatments in water. Bandte *et al.* (2010) showed that eradication of plant pathogens from AD is affected by the feedstock; ensiling of feedstocks improved pathogen kill during AD compared with using fresh feestocks and the same temperature-time treatments. These works support the view that low pH and/or release of toxic substances such as organic acids or hydrogen sulphide during AD improves kill of plant pathogens (Lukehurst *et al.,* 2010). However, Termorshuizen *et al.* (2003) found that although percolate from an anaerobic digester was toxic to *R. solanacearum* and chlamydospores of *Fusarium oxysporum f.sp. asparagi*, this was irrespective of the concentration of organic acids in the percolate, including acetic and propionic acids.

If AD feedstocks contain sufficient available nitrogen, they will generate ammonia (NH_3) which is known to be toxic to several soil-borne plant pathogens (Chun & Lockwood, 1985). A concentration of only 17 ppm was sufficient to kill mycelium and prevent germination of zoospores of *Phytophthora cinnamomi* (Gilpatrick, 1969). This is likely to occur in AD only if nitrogenous materials such as food waste or animal manures are present in the feedstocks.

8.1 Common indicator bacteria (*E. coli* and *Salmonella* spp.)

8.1.1 Impacts of mesophilic anaerobic digestion (MAD)

Around 60 to 70% of the raw sewage sludge produced in England and Wales is treated by mesophilic anaerobic digestion and thus the survival of a range of pathogens during this process is well documented. Results are supportive of a time-temperature relationship as the primary mechanisms for inactivation. However the majority of digesters in the UK are operated as completely mixed reactors. This means that some particles remain only a short time in the digester whereas others remain much longer, with the hydraulic retention time being the average retention time of all the particles. When a digester is fed by a fill and draw, or fill and spill mechanism, then some particles may leave almost immediately, a process known as short-circuiting. Drawing digestate before filling will help to eliminate short circuiting, but does not prevent all the particles exiting before the expected hydraulic retention time. These problems are not found in controlled bench-scale digestion and so their performance is generally superior to that of full-scale units.

A detailed survey of full-scale primary MAD facilities in the UK treating raw sewage sludge and that were considered to be well operated, found *E. coli* reduction to average between 1.35 and 3.36 log (UKWIR, 1999). A more in-depth 18-month survey of all the digestion sites in north-west England showed that primary digestion typically achieved a removal of 1.5 log *E. coli* with an additional contribution of 1.0 log from secondary digestion (Le *et al*., 2000). The authors also examined the effects of temperature on the secondary digestion

phase at a bench scale and observed that at 10°C a 0.5-log removal was achieved within 14 days, which increased to 1.0 log at 25°C (Le *et al*., 2001). Horan *et al*. (2004) measured inactivation of *E. coli* in both the primary and secondary stages of MAD using a 10 litre laboratory digester operated under compliant conditions and fed with raw sludge. They observed a 1.66 log removal for *E. coli* and 2.23 for *S.* Senftenberg during the primary stage. The extent of die-off was a function of the numbers of pathogens in the feed and as these increased the log removal also increased. An additional 1.70 log reduction for *E. coli* and 2.10 log reduction for *S.* Senftenberg was provided by secondary sludge digestion.

Many other studies have been undertaken to determine the survival of *Salmonella* spp. during MAD and consistent results have been obtained in the majority of these. However due to the low numbers of this organism that are usually found in sludge, investigation of its removal usually means it is necessary to inoculate the sludge artificially before digestion. Often this has been carried out with strains originally isolated from the sludge itself. For instance Carrington *et al.* (1982) used a strain of *S.* Dusseldorf isolated from activated sludge to examine its inactivation during digestion at temperatures of 35 and 48°C and with mean hydraulic retention times of between 10 and 20 days. They were able to show the specific rate of decay of this bacterium was greater at the higher temperature and greater at 15 days retention time than 10 days, although there was no difference in removal rate at retention times above 15 days. These authors also recognised the importance of the mixing and feeding regime of full-scale digesters and the role short-circuiting of the feedstock can play in increasing the numbers of viable *Salmonellae* in the digestate.

A review of *Salmonella* die-off during anaerobic treatment at plants in England and Wales found a maximum reduction of 98% (1.70 log) and this was observed in two mesophilic digesters that had mean retention times of 30 days (temperature not stated). The impact of time and temperature on die-off of *Salmonella* has been evaluated at three MAD digesters in Nashville, USA and a digester with a retention time of 9 days operated at 34°C also had a die-off of 98% (compared with 98.5% observed in this study – see Section 8.1).

Chen *et al*. (2011) examined the basis for the selection of a design retention time for MAD reactors. Using 4 litre bench reactors treating raw sludge at 35°C, they found that optimum methane production could be achieved with a shorter retention time than that required for optimum pathogen removal. Whereas the performance optimum was achieved at a retention time of 11 days, increasing this to 16 and 25 days gave removal efficiencies of 1.93, 2.98 and 3.01 $\log_{10}$ units for *E. coli* and 1.93, 2.76 to 3.72 $\log_{10}$ units for *Salmonella* spp. based on an MPN method. The effectiveness of extending retention time on pathogen removal was also shown by Forster-Carneiro *et al.* (2010) who found that the highest removal of faecal coliform colonies and *Salmonella* spp. achieved by mesophilic anaerobic digestion was obtained at a retention time of 20 days.

Jones (1976) was one of the first researchers to notice that survival of salmonellas in cattle slurry is influenced by the number of organisms originally introduced, the temperature of storage, the solids content of the slurry and the strain of salmonella involved. Survival was longest in slurries with a solids content of 5% or more. Olsen and Larsen (1987) measured the T_{90} values for both pathogenic and indicator bacteria in animal slurry during both mesophilic and thermophilic anaerobic digestion. During mesophilic digestion they found values of 2.4 days for *Salmonella* Typhimurium, 2.0 days for *S.* Dublin and 1.8 days for *E. coli*. Thermophilic digestion reduced these values to 0.7 h for *S.* Typhimurium, 0.6 h for *S.* Dublin and 0.4 h for *E. coli.*

An antibiotic-resistant strain of *Salmonella* Typhimurium was used to spike a mixture of night soil and cattle slurry to test the potential of anaerobic digestion for biogas plants in Indian

villages (Gadre *et al.,* 1986). They were able to demonstrate that this organism was totally eliminated from the digester in nine days at 25°C.

Kearney *et al*. (1993a, b) also investigated the effects of both storage and anaerobic digestion on the survival of pathogenic bacteria in beef cattle slurry. They found that the decline in viable numbers of *Salmonella* Typhimurium was temperature-dependent and declined more rapidly at 17°C than at 4°C. They also monitored a full-scale digester, fed daily and operated at 28°C with a mean hydraulic retention time (defined as the reactor volume divided by the reactor feed rate, with units of days) of 24 d. The viable numbers of *Escherichia coli* and *Salmonella* Typhimurium were reduced, and *E. coli* had a mean T_{90} value of 76.9 d.

Even at the low-temperature psychrophilic range (10 to 21°C), anaerobic digestion of swine manure in an intermittently fed, batch process operated at 20°C for 20 days was shown to reduce viable populations of indigenous indicator bacteria. Total coliforms were reduced by between 97.94 and 100%, *E. coli* by 99.67 to 100%, while *Salmonella* was undetectable (Côté *et al.*, 2006). The importance of temperature and mixing was again demonstrated by Kumar *et al*. (1999) who studied the survival of certain pathogens in anaerobic batch digesters at room temperature (18-25°C) and at 35°C. At room temperature *E. coli* and *Salmonella* Typhi survived for up to 20 days whereas at 35°C survival was only up to 10 days. Interestingly the survival time of *Salmonella* Typhi was found to increase as the solid contents of the digester were increased from 9% to 15%, confirming the observations of Jones (1976). Massé *et al*. also examined batch psychrophilic anaerobic digestion for both swine carcasses (2008) and swine manure (2011). Operated over a two-year period at 20°C, digestion for 14 days significantly decreased the concentration of faecal coliforms and *Salmonella* from about 10^6 and $10^3 CFUg^{-1}$ to undetectable levels in most samples. Even when the digestion time was reduced from 14 to 7 days the pathogen removal ability was not compromised.

The observation by Jones (1976) on the effects of solid concentration on survival led Termorshuizen *et al*., (2003) to propose that bacterial pathogens may be more persistent in vegetable, fruit and garden waste as these have a much higher solids content than cattle slurry. Consequently they monitored the inactivation of *Salmonell*a in a 300 litre reactor operated with a mixed vegetable, fruit and garden waste at a solids content of 38% (effectively a dry digestion process). The digester was spiked with 500 ml of a solution of *S.* Typhimurium at 1.7×10^8 cfu/ml and samples taken after 1, 5, 7 and 21 days of digestion. The numbers of *S.* Typhimurium were $<10^5$; $<10^5$; $<10^2$ and <1 for each time period. In the same samples the total Enterobacteriaceae (which includes the Salmonellae) were observed at 4.6×10^6; 8.6×10^3; 1.3×10^2 and 1.1×10^2.

The numerous studies that have been carried out on the survival of indicator bacteria and Salmonellae during MAD, make it clear that a single-stage MAD process has known limitations for inactivating indicator bacteria and the pathogenic Salmonellae. The inactivation efficiency is limited and only results in at best a two-log removal of faecal indicators (and a three-log removal of Salmonellae) during full-scale operation at retention times <26 days (Gantzer *et al.,* 2001; Guzmán *et al*., 2007). Performance can be significantly worse where digester mixing is not fully optimised, due to short-circuiting. Thus an additional treatment stage is required that is able to further enhance pathogen reduction and it is usual to employ a higher temperature process option at some stage in the flow train.

8.1.2 Impacts of TAnD (Thermophilic Anaerobic Digestion)

Operating anaerobic digesters at thermophilic temperatures of 55°C or greater is known to enhance the rate of the hydrolysis reaction and thus permit the digester to be operated at a higher organic loading rate (Kim *et al.,* 2003). Thermophilic anaerobic processes are also known to show a greater efficiency for degradation of organic matter, which means that they produce more biogas and a digestate with a reduced volatile solids fraction. Indeed, the digestion capacity of thermophilic digesters can be double that of comparative mesophilic processes (Krugel *et al.,* 1998; Zábranská *et al.,* 2001). This allows a reduction in the site footprint of the digester and also provides an improved specific methane yield.

Temperature is also known to be an efficient agent for inactivating microorganisms. To be effective and relatively rapid, the heat levels must be above the optimum growth temperature of the organism (Strauch, 1998). Feacham *et al*. (1983) reviewed the available literature and determined the time temperature relationship, or thermal death curve, for a number of enteric pathogens. From this they constructed a zone of safety above which, if the operating parameters were above the minimum defined by the safety zone, then the resultant biosolids (digested sewage sludge) would be pathogen free. The operating parameters were around 7 minutes exposure at 70°C; 30 minutes at 65°C; 2 hours at 60°C; 15 hours at 55°C and 3 days at 50°C. Carrington *et al*. (1998) suggested that a temperature of 70°C for 30 min or 55°C for 4 h would produce a sludge that is virtually pathogen-free. Using HACCP principles this was transcribed into a critical control point for thermophilic anaerobic digestion as: "sludge should be raised to at least 55°C for a continuous period of at least 4 hours after the last feed and before the next withdrawal. Plant should be designed to operate at a temperature of at least 55°C with a mean retention period sufficient to stabilise the sludge." Thus an increase in digester operating temperature from the mesophilic 35°C to the thermophilic 55°C would be expected to facilitate a higher pathogen inactivation. Indeed over twenty years ago Olsen and Larsen (1987) demonstrated that *Salmonella enterica* serovar Typhimurium was inactivated within 24h in a thermophilic anaerobic digester at 55°C, whereas it took weeks to achieve the same effect in its mesophilic counterpart. Consequently the potential of TAnD to provide simultaneously a pasteurization effect and an increase in energy release has been evaluated for a wide range of organic wastes.

Krugel *et al.* (1998) were interested in the potential of TAnD to aid in uprating sludge treatment facilities in the Greater Vancouver Regional District where expansion at the Annacis Island plant meant that a Class A biosolids (substantially free from Salmonella and with *E. coli* <1,000 MPN/g dry solids (DS)) must be produced in order to guarantee a local recycling route for this product. A number of options were evaluated, including pasteurization followed by MAD. A simple mathematical model that included the time/temperature relationship for pathogen die-off as well as the mixing kinetics for completely mixed digesters, suggested that the most effective route would be a multi-stage TAnD comprising one digester at 15.2 days retention time and three smaller reactors each operated at 1.5 days retention time and 55°C. This process, termed extended thermophilic anaerobic digestion, has been in operation since 1996 and producing a Class A product. The authors concluded that the US-EPA time/temperature relationship was a conservative one.

A similar approach was taken by Ahring *et al.* (2002) who uprated a 5,000 m^3 MAD process at Los Angeles to operate as a TAnD process at 55°C in order to enhance the sludge quality from its existing Class B status to a Class A status, thus providing more market outlets. After 3 months of acclimatization the biosolids always achieved the Class A standard of <1,000 MPN *E. coli* /g DS, except during those periods when the digester temperature could not be maintained.

A direct comparison of pathogen removal under the elevated temperatures at which thermophilic digesters operate compared to similar mesophilic digesters has been provided by Zábranská *et al.* (2003) who observed a 2 to 3 log removal of faecal coliforms during a MAD process but with a 4 to 5 log removal during TAnD for the same feed sludge, which had a faecal coliform concentration in the range of 10^7 to 10^9 CFU/g. Wagner *et al.* (2008) investigated *in situ* pathogen inhibition in a 750m^3 TAnD treating biowaste and were able to show that neither *Salmonella enterica* nor *E. coli* were capable of survival within the reactor for more than 2 h, and they concluded that temperature and physicochemical properties of the sludge were effective in inhibiting the survival of these microorganisms. Plym-Forshell (1995) examined a biogas plant that was fed continuously with manure from 200 dairy cows and 400 calves and young stock. A filter sac containing Salmonella was suspended in manure that was stored in a pit at an ambient temperature of between 22 and 27°C. Although Salmonella were still found in the filter sac after 35 days, viability had dropped to zero after 42 days. When the experiment was repeated and a filter sac containing Salmonella added to the digester at a temperature of 55°C, no viable salmonellas could be found after 24h.

Gannoun *et al*. (2009) examined the removal of a range of organisms in a thermophilic anaerobic digester treating abattoir waste over a range of short retention times from 0.5 to 5 days, and the removal of faecal coliforms increased as the retention time increased. There was a 1-log removal at 0.5 days and a 3 log-removal at 5 days. By contrast no *Salmonella* were observed even at the 0.5 days retention time.

8.2 Other organisms examined in this study

8.2.1 Plasmodiophora brassicae and *Spongospora subterranea*

Economically significant plasmodiophorids include *Plasmodiophora brassicae* Woronin, the causative agent of clubroot of cabbage and other brassicaceous crops; *Spongospora subterranea* (Wallroth) Lagerheim, the causative agent of powdery scab of potato and *Polymyxa betae* Keskin, which, along with a virus (*Beet necrotic yellow vein virus* (*BNYVV*)), is associated with rhizomania disease of sugar beet. *Polymyxa graminis* Ledingham, *P. betae*, *S. subterranea* and *S. nasturtii* also serve as vectors for viruses of crops including barley, wheat, potatoes, and watercress.

These organisms infect plants *via* the root system, and spread from cell to cell and between roots *via* zoospores, which are relatively short-lived. However, the lifecycle of these organisms also includes the production of resting spores (sporosori or cystosori) which can survive up to twenty years in soils without a host plant. Therefore, it is important to include resting spores in test inocula when determining survival rates.

P. betae (inoculated as soils containing cystosori) was eradicated in AD after 4 days at 55°C, based on the results of a sugar beet plant bioassay (van Rijn & Termorshuizen, 2007). This result is similar to that obtained by Dickens *et al.* (1991) for cystosori in soil/water suspensions. *P. betae* was also reduced to below detectable levels by temperature/time regimes of 8 days at 55°C or 4 days at 65°C in composting systems (Kerins unpublished).

In four studies on AD, eradication of *Plasmodiophora brassicae* (based on the results of bioassay test plants) was achieved after 21 days at 40°C or after 10 hours at 52°C, whereas the results of Engeli *et al.* (1993) indicate that the pathogen can survive in AD for 14 days at 55°C. The former results indicate that *P. brassicae* may be more temperature sensitive in AD than in composts (Noble *et al*. 2009, 2011). However, positive TaqMan PCR test results from *P. brassicae* inoculum exposed to an average temperature of 64°C for 2 days in composts with >60%w/w moisture indicated that *P. brassicae* may survive at low levels even in moist substrates (Noble *et al*. 2011). The greater sensitivity of a PCR based diagnostic

test than a plant bioassay test is confirmed by results of Staniaszek *et al.* (2001) but any result of this type may also be due to detection of nucleic acids in dead cells.

At the time of writing there has been no published work on the survival of *Spongospora subterranea* through AD. The only report on the lethal effects of temperature on the pathogen is provided by Mackay and Shipton (1983). They describe tuber treatments consisting of submersing tubers that were naturally infected with *S. subterranea* in a water bath at 55°C for 10 min. The treatment reduced the amount of powdery scab that subsequently developed when the seed tubers were planted.

8.2.2 Fusarium head blight

In the UK, four fusarium head blight (FHB) pathogens are commonly isolated from wheat crops: *F. culmorum*, *F. poae*, *F. avenaceum* and *Microdochium nivale*. Less abundant species include *F. graminearum*, *F. cerealis*, *F. tricinctum* and *F. sporotrichioides*. Worldwide, *F. graminearum* is potentially the most damaging of the FHB pathogens, in terms of both yield loss and mycotoxin production. However, under current conditions, *F. culmorum* is responsible for the greatest losses in the UK (Jennings & Turner, 1996; Turner & Jennings, 1997).

Generally, the fungi overwinter on infested crop residues (corn stalks, wheat straw, and other host plants). On infested residues, the fungi produce asexual spores (macroconidia) which are dispersed by rain-splash or wind. When conditions are warm, humid, and wet, the sexual stage of the fungi develop on the infested plant debris. If spores/conidia survived AD treatments they could initiate infections in crops. However, Seignier *et al.* (2010) found elimination of *Fusarium graminearum* after 2 days at 38°C in MAD.

8.2.3 Rhizoctonia solani

Rhizoctonia solani Kühn (teleomorph: *Thanatephorus cucumeris*) is a ubiquitous plant pathogen which probably causes more different diseases on a wider range of plants, over a larger part of the world, than any other plant-pathogenic species (Baker, 1970). *R. solani* is a member of Mycelia Sterilia which is an artificial fungal group including all fungi which are incapable of producing any spore types. *R. solani* can become associated with crops by planting into field soil or planting medium infected with mycelium or sclerotia. In the case of potatoes, it has been shown that infected seed tubers can be an important source of inoculum. The most severe disease incidence occurs on potatoes when soil-borne and tuber-borne inoculum are both present in the crop (Tsror & Peretz-Alon, 2005). If mycelia or sclerotia survive AD and become incorporated into soils where potatoes are grown there is a significant risk they would cause disease – although Seigner *et al*. (2010) reported survival of just 0.3 days at 38°C in MAD.

8.2.4 Phytophthora infestans (potato blight)

Late blight (LB) caused by the oomycete *Phytophthora infestans*, is a major disease of potato and tomato worldwide and can cause up to 100% yield losses. Blight generally overwinters in infected potato tubers which then sprout and provide the primary infections for further spread. Primary infections arise each season on plants growing from infected tubers planted with the seed, on dumps or among groundkeepers (potatoes re-sprouting from the previous years crop). Spread is *via* the asexual spores produced in their thousands in lesions on plant leaves and stems. Sporangia are dispersed by passive movement of wind, rain, or wind-blown rain. The sporangium can then germinate directly at temperatures above 15°C and quickly develops mycelial growth and subsequent sporangia on leaf, stem, and fruit tissues. No evidence was identified from the literature on survival of *Phytophthora* spp. in AD processes at the time of writing.

8.2.5 Ralstonia solanacearum and Clavibacter michiganensis ssp*. sepedonicus*

Quarantine diseases of potato caused by bacteria are a particular concern in the UK. Stringent controls are in place to prevent brown rot (caused by *Ralstonia solanacearum*) and ring rot (caused by *Clavibacter michiganensis* ssp. *sepedonicus*) diseases becoming established in the UK.

Research in Germany (Seigner *et al*., 2010) showed that *Clavibacter michiganensis* ssp. *sepedonicus* (*Cms*) in homogenized naturally infected potato tubers remained viable after 6 hours retention during experimental anaerobic digestion at 37°C with cattle slurry. Furthermore, viable *Cms* could still be recovered following one and six months ambient storage of the resulting digestate. However, no viable *Cms* was recovered after 24 hours retention at 37°C. Inactivation of *Cms* during mesophilic anaerobic digestion was not concluded to be temperature triggered, but might be attributed to the production of volatile fatty acids. Turner *et al*. (1983) concluded that the related quarantine tomato pathogen, *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), was effectively reduced after 7 days anaerobic digestion at 35°C.

Kaemmerer (2009) found thermosensitivity of *Cms* in anaerobic digestate rose considerably at temperatures above 35°C, with a $\log_{10}$ decrease in population over 10 minutes for every 8°C increase in temperature between 35-55°C. *Cms* was less thermosensitive in anaerobic digestate than in experimental buffer solution. Similarly, *Cms* was found to survive in buffer at 38°C for less than 2 days but survived in macerated potato tuber tissue for 4 weeks at the same temperature (Sächsische Landesanstalt Fur Landwirtschaft, 2005). In compost, the pathogen has been found to survive for more than 2 months at temperatures below 50°C and for more than 3 weeks at 65°C. A minimum temperature of 82°C for at least 5 min was shown to completely inactivate *Cms* (Secor *et al*., 1987). *Cmm* was shown to be eradicated from naturally infected tomato seeds that were soaked in water at 52°C for 20 min or at 56°C for 30 min (Shoemaker and Echandi, 1976; Fatmi *et al*., 1991). Treatment at 51°C for 1 h was also shown to control the related pathogen *Clavibacter xyli* subsp*. xyli* in infected sugarcane seed (Ramallo and de Ramallo, 2001).

Ralstonia solanacearum has been shown to survive MAD for more than 20 days, although Ryckeboer *et al*. (2002) demonstrated that *R. solanacearum* could be reduced to below detectable limits within one day during thermophilic anaerobic digestion of source separated household wastes at 52°C. Termorshuizen *et al*. (2003) showed that *R. solanacearum* could be reduced below detection levels following 6 weeks mesophilic (maximum temperature 40°C) anaerobic digestion of vegetable, fruit and garden waste. Elphinstone (unpublished) showed reduction of *R. solanacearum* below detectable levels within 48 hours when introduced during anaerobic digestion of sewage sludge at 37°C, further indicating that factors other than temperature are important in elimination of this pathogen. However, Seigner *et al*. (2010) concluded that *Ralstonia solanacearum* could survive for at least 30 days in anaerobic digestate held at 38°C in a batch process.

In Korea, *R. solanacearum* was eliminated from recycling nutrient solution by heating to 70°C for only 3 minutes (Lee *et al*. 1998). In the laboratory the organism was eliminated in liquid culture or infected potato pieces at temperatures as low as 55°C applied for at least 10 minutes (Elphinstone, unpublished). All biovars of *R. solanacearum* were eliminated from liquid cultures by exposure to 43°C (Date *et al*., 1993). However, the bacterium survived in diseased plant residues and in soil at 43°C for 2 days and at 40°C for 5 days. Hot air treatment at 75% relative humidity of infected ginger rhizomes eliminated *R. solanacearum* when their internal temperatures were allowed to reach either 49°C for 45 minutes or 50°C for 30 minutes (Tsang and Shintaku, 1998).

8.2.6 *Streptomyces scabies*

Bacteria of the genus *Streptomyces* are commonly found inhabiting soils, and although not under quarantine controls several species cause diseases of root and tuber crops, primarily potato but also notably carrot and parsnip. Reduction in yield due to infection with *Streptomyces* is rarely severe, although the symptoms can devalue a crop by their effect on quality.

At the time of writing there was no literature reporting the survival of *Streptomyces* specifically under AD conditions, although the genus is known to have evolved mechanisms to survive under typical soil conditions in the absence of a host. Although they form spores, *Streptomyces* spp. are not considered as resistant to heat and chemical stress as either *Bacillus* or *Clostridium* spp.

Experiments on aerobic composts have shown that some *Streptomyces* spp. are capable of survival or even increase in number during composting (Rainisalo *et al.,* 2011; Xu *et al*., 2011). Although only aerobic conditions were examined in these studies, *Streptomyces* are known to survive considerable periods of anaerobic stress, and indeed must do so in their natural habitat of the soil (van Keulen *et al*. 2007). It should be noted however that investigations of this type on species pathogenic to plants have not been reported, and the extent to which *Streptomyces* remains viable under AD conditions is unknown.

8.2.7 *Weed seeds*

As with plant pathogens, there have been numerous studies examining the effect of dry heat or heat in moist systems such as composts on the viability of seeds of different weed species (Noble *et al.,* 2011). However, the sensitivity of seeds such as tomato to heat is increased by higher substrate moisture content (Rees 1970; Idelmann 2005) so that their tolerance to heat is lower in the high moisture environment of AD than in composts (Noble *et al.,* 2011). Abdullahi *et al.* (2008) also showed anaerobic digestate to be phytotoxic, with no germination of radish (*Raphanus sativus*) seeds occurring in raw digestate or in the digestate following less than 10 weeks of post-AD aerobic treatment. Ammonia released from high nitrogen feedstocks may also be toxic to weed seeds (Haden *et al.,* 2011).

In addition to AD studies, experiments conducted on seed viability in leachates, manures and slurries (Marchiol *et al*., 1999) as well as in animal digestive systems (Blackshaw & Rode 1991) may predict what would happen during AD. The seeds of pepperwort (*Lepidium sativum*), scented mayweed (*Matricaria chamomilla*), and great burnet (*Sanguisorba officinalis*) germinated without any adverse effect when treated with leachate from municipal waste compost, whereas the germination of pigweed (*Amaranthus cruentus*) seeds was influenced by even low levels of leachate (Marchiol *et al.,* 1999). This suggested a high sensitivity for toxic components of compost leachate for this species. Ozores-Hampton *et al.* (1999) showed that immature compost containing acetic acid concentrations of 1.8-2.4 g/kg suppressed the germination of several weed species.

Many seeds are adapted to survive or are even stimulated for germination in the rumen and gut of animals, which have substrates and temperatures similar to those of MAD, and digestive retention times of less than one hour for birds to over seventy hours for large ruminants such as cows (Traveset 1998). Blackshaw & Rode (1991) examined the viability of weed seeds after rumen digestion for 24 hours and an 8-week ensiling process. Small proportions of seeds of fat hen (*Chenopodium album*), wild buckwheat (*Polygonum convolvulus*), round-leaved mallow (*Malva pusilla*), *Kochia scoparia*, redroot pigweed (*Amaranthus retroflexus*) and field pennycress (*Thrapsi arvense*) were still viable after this treatment.

There have been fewer studies examining the effects of thermophilic AD or a sanitisation stage on seeds. Ryckeboer *et al.* (2002) showed that tomato seeds did not germinate after 24 hours in AD at 52°C. Bloemhard *et al.* (1992) found that the viability of seeds of six weed species was lost after 6-minute exposures to a temperature of 75°C following a 7-day pre-incubation in pig slurry. However, seeds of *Abutilon theophrasti* remained viable after this treatment. The seeds of other weed species that have been shown to survive extended periods of composting or moist heat include hairy vetch (*Vicia hirsuta*) (Eisele, 1997), common purslane (*Portulaca oleracea*), johnsongrass (*Sorghum halepense*), and spurred anoda (*Anoda cristata*) (Egley & Williams 1990).

Appendix 2. Batch reactor tests: digestate analyses

9.0 Overview

Analyses of digestate feedstocks and digested materials are shown in Table 9-1. The initial mix was slightly alkaline; the pH of the mix fell slightly during AD. Ammonia, hydrogen sulphide and methane were detected in varying amounts in all the digestates (Table 9-2).

Table 9-1 Analysis of digestate feedstocks and digested materials

Material	pH	EC, mS/cm	Dry matter, %	Organic matter, % of dry matter
Food waste	**3.8**	**3.2**	**23.5**	**96.3**
GWE Digestate	**8.4**	**55.2**	**18.1**	**73.8**
Digestate mix	**7.9**	**38.8**	**20.9**	**85.4**
After AD	**6.7**	**32.3**	**18.3**	**87.5**

Table 9-2 Gas analysis during AD

	Hydrogen sulphide, ppm	Ammonia, ppm	Methane, % v/v
Minimum	**0.5**	**40**	**0.08**
Mean	**133**	**149**	**0.13**
Maximum	**600**	**400**	**0.25**

Appendix 3. Methods for detection of test organisms

10.0 Introduction

It is crucially important that the detection methods used in monitoring survival of test organisms in AD are capable of assessing viability: although PCR tests for the presence of DNA can be used as a general screen they must be confirmed by a bioassay of some kind.

Techniques that have been used for detecting plant pathogens in AD are similar to those used for detecting plant pathogens after heat treatment in water, soil or compost. These include plant bioassays (for obligate parasites such *P. brassicae*), plating on selective media, ELISA (for viruses, including pre-enrichment of inoculum using baiting with susceptible plants) and DNA-based methods (PCR and realtime-PCR).

10.1 PCR

Recent developments in pathogen detection and quantification have largely focused on methods based on real-time DNA polymerase chain reaction (real-time PCR) technology in which target DNA sequences are amplified using specific primers. PCR assays are now available for most pathogens of interest. However, methods based on detection of DNA have been criticised because DNA can survive in dead cells so may produce false positive results. In contrast, detection methods based on RNA amplification (such as reverse transcriptase, or RT, PCR, and NASBA) can be used to quantify some live target pathogens.

The application of PCR to quantify plant pathogens in soils, composts or biowastes is growing. In Scotland, a proportion of land used to grow seed potatoes is now being routinely tested for PCN, as a requirement under statutory legislation, using real-time PCR (Kenyon *et al,* 2010). In addition, real-time PCR assays have been developed and used to predict the risk of disease from soil-borne pathogens such as *Colletotrichum coccodes* (Lees *et al*., 2010), *Rhizoctonia solani* (Peters *et al*., 2010) and *Spongospora subterranea* (van der Graaf *et al.,* 2003; van de Graaf *et al*., 2007).

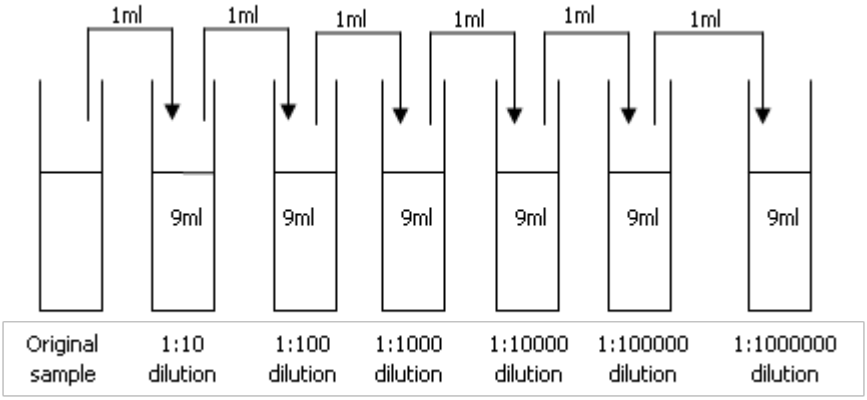
A TaqMan® BIO-PCR procedure, using DNA PCR in combination with enrichment, was shown to be a highly sensitive method for the detection of viable cells of *C. michiganensis* ssp. *sepedonicus* (*Cms*) in digester material (Kaemmerer, 2009). The TaqMan® assay of Weller *et al*. (2000) has also been used in a BIO-PCR procedure to monitor populations of *Ralstonia solanacearum* during anaerobic digestion in Germany (Seigner *et al*., 2010). This technology is easily automated (allowing high throughput sample testing) and provides semi-quantitative estimation of viable target populations. To overcome problems of PCR inhibition, Expert *et al*. (2000) found immuno-magnetic separation and DNA magnetic capture procedures potentially useful for purification and concentration of *R. solanacearum* target DNA from environmental substrates such as water, soils, sewage sludge and potato processing wastes.

10.2 MPN methods

The most probable number method is a 10-fold-dilution-based technique for determining the number of organisms within a sample.

One millilitre of the original undiluted sample is added to 9ml of a dilution media, in this case Ringers solution. The 1ml from the first dilution tube is added to the 9ml of media in the second dilution tube.

Figure 10-1 Example of an MPN dilution protocol

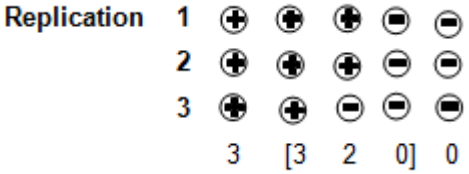


Triplicate tubes containing 9ml of the relevant growth media are then prepared. The initial three tubes have 1ml of the original sample added. The next three tubes have 1ml of the 1:10 dilution added to them. This process is repeated until the required number of triplicate tubes has been inoculated with dilutions of the initial sample.

The tubes are then incubated for a length of time and at a temperature appropriate for the required organisms to grow in the media. After this time period the tubes are examined and the number of tubes showing growth is recorded. As the dilutions are made the target organisms are diluted to extinction – that is, until they no longer produce a positive result.

The pattern of positive and negative tubes is noted and a standardised MPN table is used to determine the most probable number of organisms per unit volume of the original sample. In the example given below, the tubes with results of 3, 2, 0 are read from a standardised MPN table to determine the most probably number of organisms per unit volume of the original sample.

Figure 10-2 Example tube pattern



An excerpt of the table is below and it can be seen that 3, 2, 0 relates to 0.93, which is then multiplied by the amount of dilution in the middle tube, in this case 1:100, the amount present is 93.

Table 10-1 Excerpt of the MPN table

No. of tubes positive in first set	middle set	last set	MPN in the inoculum of the middle set of tubes
3	0	1	0.39
3	0	2	0.64
3	0	3	0.95
3	1	0	0.43
3	1	1	0.75
3	1	2	1.2
3	1	3	1.6
3	2	0	0.93
3	2	1	1.5
3	2	2	2.1
3	2	3	2.9
3	3	0	2.4
3	3	1	4.6
3	3	2	11

For *E. coli,* a lauryl tryptose broth with bromocresol purple indicator dye was used. The *Salmonella* method utilised is a two stage process (first stage MPN method) with an initial growth phase using selenite cysteine broth, followed by plating growth media onto brilliant green and xylose lysine desoxycholate agar media.

Appendix 4. Data for *E. coli* and *Salmonella* die-off during semi-continuous AD

11.0 Data tables

Table 11-1 Reactor 1 *E. coli*

Day	Dry Solids (%)	Total *E. coli* (per ml)	*E. coli* (per g.ds)	Tracer remaining	Log number remaining if just washout occurring	Log number remaining as measured	Die off (Column G-Column F)
1	3.64	9300000	255494505	100.0%	8.41	8.41	0.00
2	3.64	9300000	255494505	76.7%	8.29	8.41	-0.12
3	3.64	9300000	255494505	73.3%	8.27	8.41	-0.13
6	3.85	930000	24155844	28.4%	7.86	7.38	0.48
9	3.85	240000	6233766	21.8%	7.75	6.79	0.95
12	3.88	93000	2396907	19.3%	7.69	6.38	1.31
15	3.85	93000	2415584	17.0%	7.64	6.38	1.25
20	3.64	43000	1181319	16.0%	7.61	6.07	1.54
25	3.48	43000	1235632	13.8%	7.55	6.09	1.45
30	3.11	43000	1382637	9.7%	7.39	6.14	1.25

Table 11-2 Reactor 2 *E. coli*

Day	Dry Solids (%)	Total *E. coli* (per ml)	*E. coli* (per g.ds)	Wash out%	Log number remaining if just washout occurring	Log number remaining as measured	Die off (Column G-Column F)	Average (H)	Average (F)
1	3.44	9300000	270348837	100%	8.43	8.43	0.00	0.00	8.42
2	3.44	4300000	125000000	77%	8.32	8.10	0.22	0.05	8.25
3	3.44	4300000	125000000	73%	8.30	8.10	0.20	0.03	8.25
6	4	2100000	52500000	28%	7.89	7.72	0.17	0.32	7.55
9	4	430000	10750000	22%	7.77	7.03	0.74	0.84	6.91
12	3.85	93000	2415584	19%	7.72	6.38	1.33	1.32	6.38
15	4.06	150000	3694581	17%	7.66	6.57	1.09	1.17	6.48
20	3.6	93000	2583333	16%	7.64	6.41	1.22	1.38	6.24
25	3.49	24000	687679	14%	7.57	5.84	1.73	1.59	5.96
30	3.29	24000	729483	10%	7.42	5.86	1.55	1.40	6.00

Table 11-3 Reactor 1 *Salmonella* spp

Day	DS	Total *Salmonella* (per ml)	*Salmonella* (per g.ds)	Wash out%	Log number remaining if just washout occurring	Log number remaining as measured	Die off (Column G-Column F)
1	3.64	9300000	255494505	100%	8.41	8.41	0.00
2	3.64	9300000	255494505	77%	8.29	8.41	-0.12
3	3.64	4300000	118131868	73%	8.27	8.07	0.20
6	3.85	930000	24155844	28%	7.86	7.38	0.48
9	3.85	930000	24155844	22%	7.75	7.38	0.36
12	3.88	750000	19329897	19%	7.69	7.29	0.41
15	3.85	240000	6233766	17%	7.64	6.79	0.84
20	3.64	9300	255495	16%	7.61	5.41	2.20
25	3.48	300	8621	14%	7.55	3.94	3.61
30	3.11	250	8039	10%	7.39	3.91	3.49

Table 11-4 Reactor 2*Salmonella* spp

Day	DS	Total *Salmonella* (per ml)	*Salmonella* (per g.ds)	Wash out%	Log number remaining if just washout occurring	Log number remaining as measured	Die off (Column G-Column F)	Average (H)	Average (F)
1	3.44	9300000	270348837	100%	8.43	8.43	0.00	0.00	8.42
2	3.44	4300000	125000000	77%	8.32	8.10	0.22	0.05	8.25
3	3.44	2400000	69767442	73%	8.30	7.84	0.45	0.33	7.96
6	4	430000	10750000	28%	7.89	7.03	0.85	0.67	7.21
9	4	430000	10750000	22%	7.77	7.03	0.74	0.55	7.21
12	3.85	240000	6233766	19%	7.72	6.79	0.92	0.66	7.04
15	4.06	93000	2290640	17%	7.66	6.36	1.30	1.07	6.58
20	3.6	4300	119444	16%	7.64	5.08	2.56	2.38	5.24
25	3.49	430	12321	14%	7.57	4.09	3.48	3.55	4.01
30	3.29	2400	72948	10%	7.42	4.86	2.55	3.02	4.38

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