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A laboratory study of survival of selected microorganisms after heat treatment of biowaste used in biogas plants

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Abstract

The aim of the study was to assess the effect of pasteurisation, as set by the European regulation EC 1774/2002, on selected pathogens and indicator organisms.

Unpasteurised substrate (biowaste), including animal by-products from a full-scale biogas plant was heat treated under laboratory conditions at 70 °C and 55 °C for 30 min and 60 min.

Heat treatment at 55 °C for 60 min was not sufficient to achieve a hygienically acceptable product. Heat treatment at 70 °C for 30 min and 60 min was effective in reducing pathogenic bacteria, *Ascaris suum* eggs, Swine vesicular disease virus and indicator organisms. However, this level of pasteurisation will still not reduce the quantity of Clostridia spores, or completely inactivate heat-resistant viruses such as Porcine parvovirus or *Salmonella* phage 28B.

The results still give cause for some concern regarding the use of digested residue from biogasplants in agriculture.
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1. Introduction

In full-scale biogas plants (BGP) in Sweden, different kinds of biowaste, though not sewage sludge, are used to produce biogas. Animal by-products (ABP) such as manure, slaughter by-products, blood, lipids and other biowaste, e.g. biological household waste separated at source and waste from food industries, can contain pathogens infectious to humans and animals. The digested residue is used as a fertiliser and soil improver, and must therefore be hygienically safe, to avoid spreading diseases through the environment to all forms of animal life and humans. Hygienically safe or acceptable is here defined as indicator bacteria and salmonella being below detectable limits with the methods used in this study or with other similar standard methods.

At the time of the present study, Swedish law requires BGPs that use animal waste to pasteurise the incoming substrate at 70 °C for 60 min before digestion, to ensure a hygienically acceptable product. In May 2003, a new European regulation (EC 1774/2002) concerning ABPs was implemented, replacing laws set by the member states of the EU that previously regulated the use of animal waste. The EC-regulation divides ABPs into three categories, depending on the expected degree of pathogenic contamination. Category 1 material, which could contain

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prions, must be incinerated. Category 2 material, including carcasses (of animals other than those in category 1 material), must be sterilised (133 °C at 3 bar for 20 min) before further treatment in a BGP. Manure is included in Category 2 material, but it is allowed to be used in BGPs without sterilisation, if not deemed contagious by the authorities. Category 3 material includes ABPs from healthy animals approved for human consumption, and requires pasteurisation at 70 °C for 60 min before use in a BGP, if it is kept separate from material in Categories 1 and 2. As Category 3 material and manure may contain agents that are potentially infectious for people and animals, it is important that the pasteurisation process is sufficiently thorough to kill pathogens. The substrate used in BGPs is rarely investigated in terms of pathogenic microorganisms and their reduction after heat treatment. The difference between this substrate and other media is the heterogeneity and variation of the mixture and the potentially rather large (maximum 12 mm) particles. The effect of pasteurisation can be studied using pathogenic microorganisms or indicator bacteria or both. *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter coli*, *C. jejuni* and verotoxin-producing *Escherichia coli* O157 (VTEC) have all been isolated from substrate in full-scale BGPs. All are zoonotic agents and important bacteria causing infectious disease amongst humans and animals and many are resistant to environmental conditions.

The aim of the present laboratory study was to assess the effect of heat treatment at 70 °C on the survival of selected pathogens and indicator organisms in substrate from a large-scale BGP in Sweden. Heat treatment at 55 °C under similar conditions was also investigated due to the well-founded commercial interest in lower treatment temperatures.

We tested the microorganisms described below, as well as indigenous indicator bacteria such as coliforms, thermotolerant coliforms, presumptive *E. coli* and enterococci in survival trials. The substrate was heat-treated and the survival of the inoculated pathogens was determined after 15 (only for *A. suum*), 30 min and 60 min.

Clostridium perfringens is commonly found in substrate from BGPs and was chosen here as an indicator for spore-forming bacteria (Carrington, 2001). Porcine parvovirus (PPV) causes reproductive failure in swine and was selected as a viral indicator (Lund et al., 1996) due to its high thermo-resistance (Haas et al., 1995; Kim et al., 2000). Swine vesicular disease virus (SVDV) is not as resistant to heat as PPV (Herniman et al., 1973; McKercher et al., 1980), but has been used in inactivation studies regarding slurry (Turner et al., 1998). SVDV is a picornavirus and belongs to the genus *Enterovirus*, together with Bovine and Porcine enterovirus (King et al., 2000). In sanitation stipulations for biogas reactors, picornavirus is proposed as an indicator (Lund et al., 1996). Various types of bacteriophages have been suggested as viral indicators and indicators of faecal contamination (Havelaar et al., 1991). They are similar to viruses in structure, but easier and cheaper to analyse and harmless to all animals. *Salmonella* typhimurium phage 28B (Lilleengen, 1948) has been successfully added to sewage and used to trace leakage from sewage to groundwater (Johansson et al., 1998; Carlander et al., 2000) and has also been used as a process evaluator for liquid composting (Eller, 1995). However, it has not previously been compared with viruses with respect to heat resistance. *Salmonella* phage 28B does not occur naturally in either environmental samples or faeces. *A. suum* eggs are one of the most heat-resistant parasitic ovas, and are therefore suitable as an indicator of parasite survival (Feachem et al., 1983).

2. Methods

2.1. Substrate

The substrate used in this study was untreated, mixed biowaste from a large-scale BGP in Sweden, consisting of waste from food industries, biological household waste separated at source, and Category 3 ABPs such as manure, blood, fat, etc. The substrate was collected in clean vessels from a tap on the homogenisation tank before pasteurisation at the BGP. It was then chilled in a water bath (+8 °C) before transportation in a cold-box to the National Veterinary Institute (SVA), Uppsala, where it was kept in a refrigerator (+4 °C) at SVA before the pasteurisation trial started within 24 h. Because several trials were performed, there were several batches of substrate; hence, the content and subsequently the structure of the substrate may have varied, as raw material used in large-scale BGP normally varies over time.

An analysis was performed before every pasteurisation trial in order to check for pathogens in the original substrate and to check that no phage was present in the substrate to interfere with the *Salmonella* serovar typhimurium type 5 host bacteria.

The supplier of media in this study was SVA, unless otherwise stated.

2.2. Bacterial strains

Pathogenic bacteria inoculated into the substrate were from the strain collection at SVA: *Salmonella enterica* subsp. *enterica* serovar typhimurium, Culture Collection, University of Gothenburg (CCUG) 31969, *L. monocytogenes* CCUG 15527, *E. coli* O157 CCUG 8018 and *C. jejuni* CCUG 11284. The bacterial strains were stored at −70 °C. They were streaked onto blood agar plates and incubated at 37 °C for 24 h, before enrichment in serum broth at 37 °C for 24 h. The final concentration of bacteria in the suspension was approx 10^8 CFU ml^{-1}, except for *C. jejuni*, which had a concentration of approx 10^7 CFU ml^{-1}. The concentration was determined based on 10-fold dilutions in buffered peptone water and colony counts on agar media, as described under Bacterial analysis, except

for *L. monocytogenes* for which horse blood (5%) agar plates were used. Indicator bacteria enumerated were indigenous faecal bacteria such as coliforms (37 °C), thermotolerant coliforms (44 °C), presumptive *E. coli*, enterococci and *C. perfringens*.

2.3. Virus and cells

The PPV strain 893/76 originally isolated at the Danish Institute for Food and Veterinary Research, Lindholm, Denmark, was grown in PK-15 (pig kidney) cells, American Type Culture Collection (ATCC) CCL33, using as cell culture medium (CCM) Eagle's minimal essential medium (MEM) (Eagle, 1959), supplemented with non-essential amino acids (acids (L-Alanine 8.9 mg l^{-1}, L-Asparagine monohydrate 15 mg l^{-1}, L-Aspartic acid 13.3 mg l^{-1}, L-glutamic acid 14.7 mg l^{-1}, L-proline 11.5 mg l^{-1}, L-serine 10.5 mg l^{-1}) and containing 4% foetal bovine serum (FBS). The suspension was frozen and thawed twice, clarified by compressed air filtration through 3.0 μm and 0.45 μm filters (Millipore, Billerica, MA, USA), aliquoted and stored at −70 °C. The final titre of PPV suspension was 6.9 $\log_{10}$ tissue culture infectious dose $(TCID)_{50}$ per 50 μl. The titre was determined as described under Virus analysis.

The SVDV strain 27/72 was grown in IB-RS-2 (pig kidney) cells (both obtained from Pirbright Laboratory, Institute for Animal Health, UK) using Eagle's MEM containing 2% FBS. After full cytopathogenic effect, the virus-infected cells and supernatant were frozen and thawed twice, and clarified by centrifugation at 2500×g for 20 min. The virus suspension was aliquoted and stored at −70 °C. The final titre of SVDV was 8.1 $\log_{10}$ $TCID_{50}$ per 50 μl, determined as described under Virus analysis.

2.4. Propagation of Salmonella typhimurium phage 28B

Salmonella typhimurium phage 28B and its host *Salmonella enterica* serovar typhimurium type 5 (Lilleengen, 1948) were obtained from the Swedish Institute for Infectious Disease Control, Solna. The bacteriophage was propagated in Nutrient Broth (Oxoid) against its host strain to a concentration of approx 10^{10} PFU ml^{-1}. Determination of the concentration was performed in the same way as described under Analysis of *Salmonella* phage 28B. The bacterial host was killed by adding chloroform (10 ml l^{-1}). The phage solution was then centrifuged for 30 min at 4300×*g* and filtered through 0.45 μm membrane filters to remove cell debris.

2.5. Parasites

A. suum eggs were obtained from roundworms in fattening pigs at the slaughterhouse in Uppsala, Sweden, via the Department of Parasitology, SVA. The *A. suum* eggs were stored in 0.1 M H_2SO_4 at 4 °C before the pasteurisation trial.

2.6. Inoculation of pathogens and pasteurisation trial

The substrate was distributed into 250 ml glass bottles. The bacterial pathogens were inoculated into the substrate to a final concentration of approx 10^5 CFU g^{-1} of each bacterium. One bottle with a mix of bacterial pathogens was used for each temperature–time combination and one bottle was not heated and analysed as positive control. Indicator bacteria were not inoculated, whereas indigenous indicator bacteria were enumerated in the analysis. The viral agents were treated in separate bottles at the desired temperature. The inoculation ratio for the viruses was 1:10 and the theoretical initial virus mean concentration was 7.2 $\log_{10}$ $TCID_{50}$ g^{-1} for PPV and 8.4 $\log_{10}$ $TCID_{50}$ g^{-1} for SVDV. The phage was inoculated into the substrate to a final concentration of approx 10^8 PFU g^{-1}. *A. suum* eggs were separately heat-treated in substrate kept in small tea-bag-sized polyethylene bags with a pore size of 0.20 μm. Each bag contained about 10^4 eggs. The concentrations of inoculated pathogens in this study were based on numbers of indicator bacteria normally found in BGP in previous studies (Herniman et al., 1973; Haas et al., 1995) and a worst-case scenario.

Heat treatment was performed in a water bath at 70 °C or 55 °C, with continuous mixing in the bottles. The temperature was continuously controlled with a thermosensor in the substrate, and timekeeping began when it reached the desired level (70 °C or 55 °C) in the whole substrate. The temperature was reached after 14–20 min of heating in the water bath, and thus it simulated the actual process in the biogas plant. Samples for analysis were taken directly after inoculation and after 30 min and 60 min. In addition, analysis of *A. suum* eggs was also performed after 15 min.

Substrate samples containing pathogenic bacteria, viruses, *A. suum* eggs (in bags) or *Salmonella* phage 28B were kept in bottles similar to the ones used in the experiment, at approx 20 °C during the trial, as control samples. The purpose of the controls was to study the effect of the substrate on the reduction of the organisms. Additionally, a suspension of the two viruses was kept in CCM, while bags with *A. suum* eggs were kept in 0.1 M H_2SO_4 at approx 20 °C as controls. The pasteurisation trial and analysis of the pathogens and indicator organisms were repeated three times.

2.7. Bacterial analysis

S. typhimurium, *C. jejuni*, *E. coli* O157, *L. monocytogenes* and *C. perfringens* were analysed according to the methods described in Sahlström et al. (2004). Suspected colonies of *L. monocytogenes* in the positive control samples were confirmed by Gram staining rendering visible Gram-positive colonies including *L. monocytogenes*. Counting of indicator bacteria was performed after 10-fold dilutions in peptone–water according to NMKL 91:2:1988. The NMKL methods used were: for enterococci, NMKL 68:2:1992, where samples were plated onto enterococcus

agar according to Slanetz and Bartley (Oxoid) and incubated at 44 °C for 48 h; for coliform bacteria, NMKL 44:4:1995 utilising violet red bile agar (Difco) and incubated at 37 °C for 24 h; and for thermotolerant coliform bacteria and presumptive *E. coli*, NMKL 125:3.1996. The same agar as for coliform bacteria was used, but it was incubated at 44 °C for 24 h. Confirmation of coliform bacteria was performed by gas production in Brilliant Green broth. Confirmation of thermotolerant coliforms was performed in lactose tryptone lauryl sulphate broth (LTLSB) (Oxoid). For examination of presumptive *E. coli*, Kovaćs reagent for detecting indole was added to the inoculated LTLSB tubes.

2.8. Virus analysis

Samples were centrifuged in a microcentrifuge at 7000×*g* for 2 min, as this has been shown to ensure sufficient virus recovery to obtain an adequate initial virus titre of the spiked substrate, with regard to SVDV (Turner et al., 1999). To reduce cytotoxicity where appropriate for analysis of SVDV, the centrifuged samples were filtered through 0.45 μm sterile filters (Millipore, Billerica, MA, USA), followed by treatment with a PD-10 desalting column containing Sephadex™ G-25 Medium (GE Healthcare, Chalfont St. Giles, UK). This treatment was shown not to reduce the virus titre. Cytotoxicity and viral interference studies were performed using substrate treated as above. Cytotoxicity tests were performed by diluting the treated substrate 3-fold in cell culture medium, inoculating onto IB-RS-2 cells, and observing cytotoxicity for up to 4 days. As control cell culture medium was used. The viral interference studies were performed by titrating SVDV in the 3-fold dilutions of the treated substrate, inoculating onto IB-RS-2 cells and observing the virus titres after 4 days. SVDV titrated in cell culture medium was used as control.

Titration of samples and titre calculations were performed using a $TCID_{50}$ quantal assay (Kärber, 1931).

Cells and CCM used were as described under Virus and cells, with an extra addition to the CCM of 0.75 $\mu g\ ml^{-1}$ of fungizone (Bristol–Myers Squibb, NY, USA). CCM without serum was used as sample dilution medium and as a negative control. Samples from each treatment were diluted 10-fold (or 3-fold where a low virus titre was expected) and added to microtitre plates containing cells, with eight 50-μl replicates per dilution. After 4 days (SVDV) or 7 days (PPV) of incubation at 37 ± 1 °C in $5 \pm 1\%$ CO_2 in a humidified atmosphere, the cells in the microtitre plates were analysed microscopically to determine the cytopathogenic effect. For PPV, a specific immunoperoxidase test (Emmoth et al., 2004) was used to visualise the infected cells prior to microscoping.

2.9. Analysis of Salmonella phage 28B

Samples of 10 g were combined with 90 g buffered peptone–water and mixed in a Stomacher. Counts were made after 10-fold dilutions in sodium chloride. *Salmonella* phage 28B was analysed by a double-agar layer method (Adams, 1959). The host strain *Salmonella* serovar *typhimurium* type 5 was cultured in Nutrient Broth at 37 °C for 4 h. A mixture of 1 ml sample, 1 ml cultivated host and 3 ml soft agar (70% Blood agar base (CM55, Oxoid) and 30% Nutrient Broth) was spread on a dry CM55 agar plate and incubated at 37 °C for 18 h. Clear zones (plaques) were counted as PFU.

2.10. Analysis of A. suum ova

After pasteurisation, the bags with *A. suum* eggs were placed in 0.1 M H_2SO_4 at approx 20 °C to mature. After 4 weeks, 1000 eggs from each bag were counted, and scrutinised for developed larvae. The number of developed larvae among the heat-treated eggs was compared with the number of developed larvae in the unpasteurised control, which was kept in 0.1 M H_2SO_4 at approx 20 °C.

3. Results

3.1. Bacteria

The pathogenic bacteria that were inoculated and analysed in the trial could not be detected in the original substrate. No inoculated pathogenic bacteria or indicator bacteria, except for *C. perfringens*, could be found after heat treatment at 70 °C for 30 min or 60 min. *S. typhimurium* and *E. coli* O157 were once detected after 30 min at 55 °C, but after 60 min at 55 °C, no inoculated pathogenic bacteria could be found. The reduction of the indicator bacteria at 55 °C and 70 °C is presented in Table 1. The number of *C. perfringens* (mean of 4.8 $\log_{10}$ in the original substrate) was not significantly (Students' *T*-test; $P < 0.05$) affected by heat treatment (mean 4.4 $\log_{10}$ after 60 min at 70 °C).

3.2. Viruses

PPV or SVDV could not be detected in the original substrate from the BGP. Virus titres before and titre reductions after treatment at 55 °C and 70 °C are presented in Table 2. PPV was reduced by a mean of 3.2 (min 3.0 and max 3.3) $\log_{10}$ units after 60 min at 70 °C. SVDV was inactivated to below the detection level after 30 min at 70 °C, which gave a mean reduction of $\geqslant 6.1$ (min $\geqslant 5.6$ and max $\geqslant 6.4$) $\log_{10}$ units. For the three SVDV experiments at 70 °C, the viral interference studies showed that the lowest dilution of the treated substrate that could be used for the titre reduction calculations was 1.7, 1.8 and 2.4 $\log_{10} TCID_{50}\ g^{-1}$, respectively, due to somewhat different cytotoxicity and viral interference of the treated substrates. Thus, the mean detection limit concerning SVDV for the substrate was found to be 2.0 $\log_{10} TCID_{50}\ g^{-1}$, based on the results from the viral interference study.

Table 1
Content of indigenous indicator bacteria in the substrate before and after 30 min and 60 min (pre-heating time 14–20 min) in 55 °C and 70 °C, respectively

Bacteria	Before heat treatment ($n = 6$)[a]		55 °C 30 min ($n = 3$)		55 °C 60 min ($n = 3$)		70 °C 30 min ($n = 3$)		70 °C 60 min ($n = 3$)	
	min	max	min	max	min	max	min	max	min	max
Enterococci	4.1	5.4	0	3.1	0	3.7	0	0	0	0
Coliforms 37 °C	4.3	5.6	0	2.7	0	0	0	0	0	0
Coliforms 44 °C	4.1	5.4	0	2.9	0	0	0	0	0	0
Presumptive *E. coli*	4.1	5.4	0	2.7	0	0	0	0	0	0
C. perfringens	3.9	5.4	4.0	4.9	3.9	5.0	4.0	4.4	4.0	4.6

The figures ($\log_{10}$ cfu g^{-1}) represent the minimum and maximum amount of bacteria from the results of the three trials (n = number of samples).
[a] Includes samples from both 55 °C and 70 °C trials.

Table 2
Reduction of PPV, SVDV and *Salmonella* phage 28b (Ph 28b) after heat treatment

Virus	Before heat treatment ($n = 6$)		55 °C 30 min ($n = 3$)		55 °C 60 min ($n = 3$)		70 °C 30 min ($n = 3$)		70 °C 60 min ($n = 3$)	
	min	max	min	max	min	max	min	max	min	max
PPV	6.4	7.6	0.7	1.5	1.0	2.1	2.2	3.1	3.0	3.3
SVDV	7.3	8.8	1.1	1.6	1.5	2.1	$\geqslant$5.6[a]	$\geqslant$6.4[a]	$\geqslant$5.6[a]	$\geqslant$6.4[a]
Ph 28b	7.8	7.9	nd	nd	nd	nd	0.0	0.1	0.2	0.2

Initial content of PPV, SVDV and Ph 28b in the spiked substrate and reductions after 30 min and 60 min (pre-heating time 14–20 min) at 55 °C or 70 °C are presented. The figures for spiked substrate (virus $\log_{10}$ $TCID_{50}$ g^{-1} and phage $\log_{10}$ pfu g^{-1}) represent the minimum and maximum initial amount of viruses and phage and include spiked samples from both 55 °C and 70 °C trials ($n = 6$). The figures after each heating step represent the minimum and maximum $\log_{10}$ reductions from the three trials (n = number of samples). nd = not determined.
[a] Virus titre fell below detection limit.

As regards the control samples in substrate, a mean reduction of $0.5\log_{10}$ units for PPV and of $0.2\log_{10}$ units for SVDV was observed. This reflects the inactivating capacity of the substrates from the BGP, as the controls in cell-culture medium were stable (results not shown).

3.3. *Salmonella phage 28B*

According to a check performed three times, in which substrate without added phages was analysed, no phage was present in the substrate to interfere with the *Salmonella* serovar *typhimurium* type 5 host bacteria. No reduction of *Salmonella* phage 28B was observed after treatment at 70 °C for 30 min. After 60 min at 70 °C, the *Salmonella* phage 28B had not significantly decreased (Students' T-test, $P < 0.05$) from a mean of $7.9\log_{10}$ PFU g^{-1} before heat treatment to a mean of $7.7\log_{10}$ PFU g^{-1} after heat treatment.

3.4. *A. suum*

No developed larvae of *A. suum* could be detected in the samples treated for 15 min, 30 min or 60 min at 55 °C and at 70 °C. The development of larvae in the control samples kept at approx 20 °C in 0.1 M H_2SO_4 was 88.5%. The development of larvae was not affected in the unpasteurised control in the substrate.

4. Discussion

Pasteurisation at 70 °C for 30 min and 60 min is enough to ensure a hygienically safe product, as regards the *A. suum* eggs and the bacteria included in this study, except for *C. perfringens*. However, *Salmonella* phage 28B was scarcely affected, and PPV was not reduced sufficiently at 70 °C for 60 min. Carrington (2001) reports an average level of 10^4 g^{-1} enteroviruses in raw sewage sludge. Based on those figures, Carrington (2001) suggests a $4\log_{10}$ reduction of virus, which would ensure virus reduction to undetectable levels, hence a sufficient hygienic level of sewage sludge. In a similar study, Lund et al. (1996) demonstrated a reduction in PPV of $1.4\log_{10}$ units after a 60 min pasteurisation at 70 °C and these authors opposed the requirement of a $4\log_{10}$ reduction as too high for such a heat-resistant virus as PPV. However, in the present study, PPV reduction reached a minimum of $3.0\log_{10}$ units after 60 min pasteurisation at 70 °C. Depending on the initial amount of virus in the substrate, a reduction of $3\log_{10}$ may be too low to achieve an adequate hygienic level in the digested residue, according to Carrington (2001). However, the EU Commission has now proposed a reduction of $3\log_{10}$ units for validation of other processes that could replace the 60 min pasteurisation at 70 °C (SANCO/2632 and 2634/2005) for ABPs and manure.

However, in contrast to bacteria, viruses are unable to grow and increase in number outside their host. One can only speculate about the possible amount of virus in substrate in a BGP. Taking into account the new regulation (EC 1774/2002), which divides ABPs into three different categories with regard to the risk of contamination, the possibility of high virus quantities in Category 3 material, which is used as substrate at BGPs, should be low. However, there is still a risk of subclinically infected animals.

For example, PPV infection is widespread in the swine population and the virus is excreted in the faeces (Klingeborn, 2004). In addition, concern about cross-contamination or other residual infectivity in Category 3 material was recently addressed by Böhm (2004). His results show that bovine parvovirus in slurry only reaches a mean reduction of $2.6\log_{10}$ after 60 min heat treatment at 70 °C, which indicates that also other heat stable viruses such as circo- and caliciviruses would survive.

Owing to their properties of being easy and safe to analyse and handle, bacteriophages ought to be excellent indicators of viral contamination. *Salmonella* phage 28B was almost unaffected by treatment at 70 °C for 60 min. Compared with the reduction in the two other viruses (SVDV and PPV), *Salmonella* phage 28B proved considerably more resistant to heat. It might therefore be too conservative an indicator organism for evaluating a one-hour pasteurisation process at 70 °C. For prolonged processes such as composting, however, *Salmonella* phage 28B can be used as an indicator for human and animal viruses (Eller, 1995). For shorter processes, other less heat-resistant bacteriophages could be more suitable (Moce-Llivina et al., 2003).

In agreement with findings by Feachem et al. (1983), *A. suum* eggs were inactivated after 15 min at 55 °C. However, heat treatment for 30 min and 60 min at 55 °C did not ensure inactivation of all indicator bacteria, viruses and the phage. Even some pathogenic bacteria, such as *E. coli* O157 and *S. typhimurium*, survived the heat treatment at 55 °C for 30 min. Amongst indicator bacteria, enterococci are particularly resistant to heat, as demonstrated earlier by for example Kearns et al. (1995).

Owing to their spore-forming capacity, *Clostridium* spp. are extremely heat-resistant and consequently require sterilisation (130 °C at 3 bar for 20 min) to be inactivated. Such a treatment is an economically unreasonable requirement for commercial BGP in practice. In this study, *C. perfringens* was analysed as an indicator for other *Clostridium* spp. that cause severe disease amongst cattle. Moreover, *Clostridium tyrobutyricum* can cause problems in the dairy industry, e.g. late blowing of cheese (Dasgupta and Hull, 1989). The present study indicates that problems with spore-forming clostridia may increase if *Clostridium* spp. are spread through digested residue on grassland grazed by dairy cows. Such application is prohibited 3 weeks before grazing according to Article 22:1c in EU regulation 1774/2002, if ABPs are included in the substrate used at the BGP. However, bacterial spores are able to survive far longer than 3 weeks. The risk with such use of digested residue could be a spread of spore-forming bacteria causing diseases as blackleg, botulism, tetanus and anthrax to areas still free from these diseases.

In this study, the effect of pasteurisation in full-scale BGPs under controlled laboratory conditions was evaluated. The substrate used was obtained from a full-scale BGP and the aim was to mimic the full-scale conditions as closely as possible. As described previously, the content of the substrate may have varied somewhat, as the substrate in full-scale BGPs tends to vary according to availability and season. Despite that, the pasteurisation must in all circumstances fulfil the need for adequate hygienisation of the substrate. The results from the three different trials performed gave similar results, which demonstrate that pasteurisation works as expected at 70 °C, but it does not always fulfil the criteria to reduce all pathogens at 55 °C.

As mentioned, EU regulation 1774/2002 is proposed to be amended by SANCO/2632 and 2634/2005, which allow other processes demonstrated to achieve the same minimised risk as 70 °C for 60 min to be used in treatment of ABPs and manure. The method used here to evaluate pasteurisation could also be used as one way to evaluate other combinations of time and temperature before such methods can eventually be accepted for treatment of Category 3 material according to the imminent EC-regulation (2006).

Ward et al. (1999) showed that *Salmonella* spp. and faecal coliforms in thoroughly pasteurised substrate were not able to regrow within the solids if the substrate was not re-contaminated from the surroundings. The heat-up time in this laboratory study mimics the time in the heat-exchanger in a full-scale biogas plant. However, there are other differences between a full-scale BGP and the almost ideal conditions in a laboratory study. Achieving a uniform temperature level in the whole substrate at full-scale might not be as easy. This was also evident in the present laboratory-scale study, because uneven temperatures were observed within the bottles during heating of the substrate. Safety margins should therefore be considered, and 60 min pasteurisation at 70 °C would be the minimum acceptable. Our results still raises some concerns regarding the use of digested residue from BGPs in agriculture.

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