Impact of temperature-time combinations on enteric bacteria in separated solids from pig manure

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Introduction

In Brittany (France), due to a high pig concentration and the limited availability of agricultural land, it is becoming increasingly necessary to treat the manure produced to reduce the nutrient composition ahead of land spreading. Amongst such treatments, mechanical separation is sometimes used to reduce the phosphorus load in the liquid. This process leads to the formation of a solid, separated pig solids (SPS), which is stored several weeks before being exported from the farm. This type of by-product, although useful as an organic fertiliser, does carry a microbiological risk because of the possible presence in the raw manure of pathogenic micro-organisms such as *Salmonella*, *Campylobacter, Yersinia enterolitica* [1] or *Cryptosporidium* [2].

A raised temperature is considered to be the most important factor for a successful inactivation of pathogens but an adequate temperature to ensure their destruction depends on (i) the retention time (ii) the type of micro-organism, and (iii) on the subsequent process of composting itself (whether forced or natural aeration and the frequency of turning). According to Golueke [3], a temperature maintained at 55ºC for three days in the windrow is sufficient to ensure an adequate "hygienisation" of composted sludge. This temperature-time combination is generally retained as a benchmark for hygienisation. The three days recommended by the US EPA for the systems with forced aeration, is increased at 15 days for the systems with natural aeration, if the compost is turned at least five times during this period.

Sanitary concerns linked to animal production are growing in the European Union leading to new regulations to control animal manure management. Of these, the most important are the Animal By-Products Regulations (1774/2002*)* which define all livestock manure, including SPS as category 2 material. If untreated, local land spreading is still permitted. However, once any treatment is attempted, then all material must be subjected to a minimum of 70ºC for 60 minutes and then be tested as free of *Salmonella* in 25 g (wet weight). Equivalent processes may be permitted if a similar quality of treatment is demonstrated. However, sustaining temperatures above 60ºC can be difficult to guarantee in a biological compost system especially if done outside in heaps or windrows as is often the case.

The aim of this study was to compare the hygienic impact of different temperature-time combinations on fresh SPS resulting from the centrifugation of the raw liquid piggery manure. Laboratory scale trials were carried out to investigate the effect of three process temperatures (55, 60 and 70°C) and various retention times (from 1 hour to 6 days) on bacterial indicators (*E. coli*, enterococci and spores of *C. perfringens*).

Material and methods

Experimental set-up

The effect of the temperature-time combination was carried out on fresh SPS from 3

piggeries (labelled A, B and C). Samples were taken after centrifugation: about 30 kg were homogenized and brought in to the laboratory. The moisture content was 35.9, 30.5 and 32.8 % for SPS taken from farms A, B and C respectively. The samples were then divided to obtain a series of sub samples of 50 g wet weight each which were placed into plastic bags. These bags were subsequently divided between water baths held at three different temperatures over 6 days: 55, 60 and 70ºC. The temperatures were controlled to $\pm$ 0.5°C by thermometers placed inside the SPS sample. In order to take into account the time for each sample to reach the set temperature, once the samples had been transferred to the water bath, the temperature was followed until the target value was reached. Preliminary tests showed that this lag time ranged from between 15 to 35 minutes. On reaching the set temperature, the timer was set at zero and the bags were taken and subsequently analysed at regular interval corresponding to 1h, 2h, 4h, 6h, 24h, 48h, 72h and 144 h. Each temperature-time combination was tested in triplicate. Separate control samples were placed in plastic bags at 18°C and were analyzed after 24 hours.

Enumeration of bacteria

From each bag, an amount of 10 g of SPS sample was transferred into 90 mL of peptone water (for *E. coli* and enterococci) or Tryptone Salt broth (for *C perfringens*) and then serially 10-fold diluted. *E. coli* was enumerated using 3M™ Petrifilm *E. coli* (incubated 24 h at 44°C). Detection of *E. coli* was based on enumeration of blue colonies (glucuronidase positive). Between three and five blues colonies present on Petrifilms were transferred into peptone water (incubated 24 h at 44°C). Kovacs' reagent was added to confirm *E. coli* by indole-reaction. Enterococci were enumerated on selective Slanetz–Bartley agar (Biokar, France), incubated 48 h at 37°C and subsequent confirmation on Bile Esculin Agar (Biokar, France) incubated 4 h at 44°C. Spores of *Clostridium perfringens* were enumerated according to protocol described by Sartory *et al.* [4]. After a thermal shock at 80°C for 20 min, one mL of each dilution was poured into Petri dish. Approximately 20 mL of Tryptose Sulfite medium (OXOID) added with cycloserine (0.4 g/L) (TSC agar) were then added to the dilution. After mixing, when agar had solidified, the plates were overlaid with an additional 6 to 8 ml of TSC agar and incubated in anaerobic jar 24 h at 44°C. Presumptive colonies of *Clostridium perfringens* (n = 10 to 15 per plate) which appeared as black colonies were picked off and streaked onto Columbia agar (OXOID, France). After an incubation for 24 h at 37°C, isolates were confirmed using phosphatase reagent which was applied directly onto the colonies. Colonies which developed a purple or brown colour within 3 minutes were considered acid phosphatase positive. All results were expressed as wet weight of sample.

Results

The results from the study are set out in Table 1: concentrations of indicator bacteria in the SPS taken from the three piggeries were in the range of 4.2 to 5.2 $\log_{10}$ units of bacteria or spores per gram which correspond to those observed in raw liquid manure. The importance of farm source on the persistence of bacteria in the SPS is largely insignificant as might be expected; similar results are observed. These combined clearly show the impact of the time-temperature combination on the survival of the indicator bacteria naturally present in SPS. Concentrations of enterococci and *E. coli* were reduced by 4 $\log_{10}$ units within 6 hours and 2 hours at 55 and 60°C respectively and dropped to below detectable level within 1 hour at 70°C. However, temperatures below 70ºC were not sufficient to provide a complete safe hygenisation of the SPS as *C. perfringens* was still detected even after 6 days (Figure 1). Only the temperature of 70°C was sufficient to inactivate the sporulated forms and only then if this temperature is maintained for at least 48 hours.

The survival of *E. coli* and enteroccoci were similar after 24 hours at room temperature as their concentrations did not significantly differ from those observed at T_0 (Figure 1).

However, at 55 and 60°C the behaviour of the two indicators differed as *E. coli* declined more rapidly than enterococci regardless the farm source of the SPS. *E coli* were not detected after 1 hour at 55°C while the enterococci were still detected at concentrations higher than the European regulation limit of 5 x 10^3 bacteria / g (3.7 $\log_{10}$ units / g). The inactivation of the enterococci was achieved if this temperature was maintained between 6 and 24 hours. A difference of behaviour appeared also at 60°C as enterococci were still detected at time T_x and after 1 hour (for 2 of the 3 measurements) contrary to *E. coli*.

Table 1 : average concentrations of bacteria (expressed in $\log_{10}$ units per gram of wet sample) in SPS taken from 3 piggeries (A, B and C) at T_0 (prior to experiment), T_x (nominal time zero) and after 1 to 72 h of incubation at 55, 60 and 70°C

Temp.	time	*E. coli*			Enterococci			spores of C. *perfringens*		
		A	B	C	A	B	C	A	B	C
	T0	4.7	4.3	4.2	4.9	4.6	4.8	4.8	4.7	5.2
55°C	Tx	4.3	<1.7	<1.7	4.9	4.1	4.8	-	-	-
	1h	<1.7	<1.7	<1.7	4	4.1	4	-	-	-
	6h	<1.7	<1.7	<1.7	3.3	1.6	<1.3	4.7	4.8	4.2
	24h	<1.7	<1.7	<1.7	<1.3	<1.3	<1.3	4.9	4.9	4.4
	72h	-a	-	-	-	-	-	4.6	3.9	4.6
60°C	Tx	<1.7	<1.7	<1.7	3.7	3.6	4.2	-	-	-
	1h	<1.7	<1.7	<1.7	3.7	<1.3	2.1	-	-	-
	6h	<1.7	<1.7	<1.7	<1.3	<1.3	<1.3	4.8	4.8	5.1
	24h	<1.7	<1.7	<1.7	<1.3	<1.3	<1.3	4.5	4.4	4
	72h	-	-	-	-	-	-	4.6	2.6	4.3
70°C	Tx	<1.7	<1.7	<1.7	-	<1.3	<1.3	-	-	-
	1h	<1.7	<1.7	<1.7	<1.3	<1.3	<1.3	-	-	-
	6h	<1.7	<1.7	<1.7	<1.3	<1.3	<1.3	3.6	2.6	4.2
	24h	<1.7	<1.7	<1.7	<1.3	<1.3	<1.3	1.5	1	1.4
	72h	-	-	-	-	-	-	<1	<1	<1

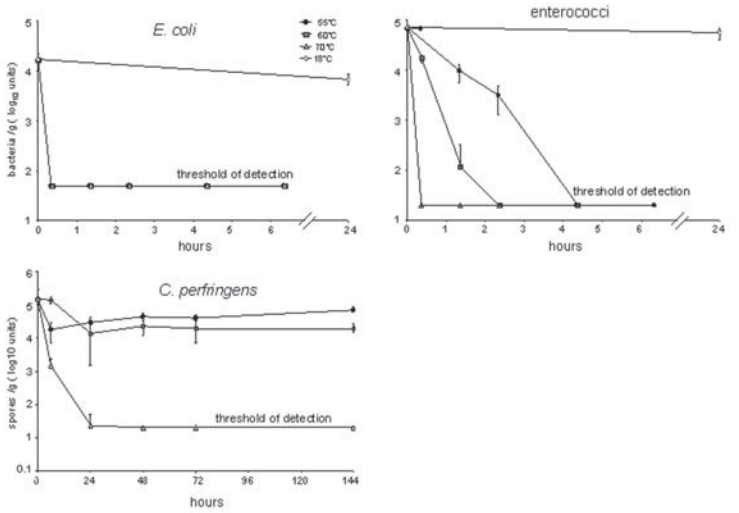
[a] no data

E. coli were destroyed very quickly even during the pre-warming phase prior to the start of the timed exposure at the set temperature. It is noted that this bacteria was not detected for 8 of the 9 measurements at T_x (Table 1) (which implied a progressive warming between 15 and 36 min). The corresponding decrease in concentration was thus 4 logarithmic units in much less than 1 hour. These results are in agreement with the data of Turner [5] who inoculated manure incubated at 55°C with *E. coli* at an initial concentration of *ca.* 10^9 bacteria /mL and who observed a reduction in the bacterial concentration of 7 to 8 logarithmic units in 1 hour.

Discussion

The process of centrifugation of raw manure alone had no effect on the concentration of enteric bacteria as the concentrations of indicators bacteria were in the same order of magnitude than those observed in raw liquid manure by several authors [6-10]. These results confirms the earlier study of Pourcher *et al.* [11] who observed no significant reduction of the concentrations of *E. coli* and enterococci after mechanical separation of SPS from 2 piggeries studied over a period of 6 months. Thus, the SPS may contain similar level of pathogen micro-organisms than raw manure. For this reason, it remains important to reduce the microbial risk of these by-products before export, for example by composting.

Figure 1 : average bacterial counts measured in SPS from Piggery A held at 18, 55, 60 and 70°C. Bars indicate minimum and maximum value of triplicates



The required time-temperature combination clearly depends on the type of bacteria considered. The higher resistance of enterococci than *E coli* observed in this study has already been mentioned by other authors [12-14]. *C. perfringens* appeared much more resistant than the two other indicators as its inactivation required at least 48 hours at 70°C: this supports the earlier results of Pourcher *et al.* [15] who observed that *C. perfringens* presented a survival higher than that of enterococci during the composting of a straw-sludge mixture. The failure to completely inactivate *C. perfringens* at 60°C in the three SPS samples confirms that the absence of detection of *E. coli* or enterococci did not systematically involve a reduction of spores or cysts of pathogenic micro-organisms. Whereas Mohaibes and Heinonen-Tanski [16] reported that keeping liquid manure for 4 hours at 55°C or 30 minutes at 70°C inactivate most pathogens, the data obtained in this study showed that the temperature of 70°C even maintained 1 hour at 70°C is not sufficient to eliminate all bacterial spores. These data illustrate the difficulty in formulating rules for the hygienisation of the manure by-products.

It is important to underline that the results of our study were obtained at a laboratory scale (using 50 g samples in water baths) and did not take into account the heterogeneity of SPS stored in large heaps at the pig farm. Indeed, Pereira-Neto *et al.* [17] and separately Pourcher *et al.* [9] reported that the temperatures within a compost pile varied greatly. Values higher than 65°C were readily reached within the core of the heap but there were zones corresponding to the surface, edges or the bottom of the heap where temperatures were lower than 40°C. In order to compensate for these cold zones, it is thus recommended to carry out mixing at minimum regular intervals. These turning operations have the further advantage of homogenizing the compost and they can re-invigorate the bacterial activity that sustains the elevated temperatures.

Conclusion

This study shows that temperatures of 55 or 60°C could have an equivalent hygienic effect to that of 70°C maintained one hour, as recommended by the European regulation when compared on the criterion of eliminating the two indicator organisms, *E. coli* or enterococci. However, temperatures of 55 and 60°C are insufficient to eliminate the spores of *C. perfringens* even if such temperatures are maintained for 6 days. One might note

that the 70°C and one hour specified under the European Animal By-product regulations are also insufficient conditions to eliminate the spores of *C. perfringens*.

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References

[1] Guan, T.Y. and Holley, R.A. (2003) Pathogen survival in swine manure environments and transmission of human enteric illness: A review. Journal of Environmental Quality 32, 383-392.
[2] Bornay-Llinares, F.J., Navarro-i-Martinez, L., Garcia-Orenes, F., Araez, H., Perez-Murcia, M.D. and Moral, R. (2006) Detection of intestinal parasites in pig slurry: A preliminary study from five farms in Spain. Livestock Science 102, 237-242.
[3] Golueke, C.G. (1983) Decision and strategies for regional resources recovery. . In: In: Biological reclamation and land utilisation of urban wastes Zucconi, F., De Bertoldi, M., Coppola, S., Ed. Vol. pp. 310-313. Napoli.
[4] Sartory, D.P., Waldock, R., Davies, C.E. and Field, A.M. (2006) Evaluation of acid phosphatase as a confirmation test for Clostridium perfringens isolated from water. Letters in Applied Microbiology 42, 418-424.
[5] Turner, C. (2002) The thermal inactivation of E-coli in straw and pig manure. Bioresource Technology 84, 57-61.
[6] Chinivasagam, H.N., Thomas, R.J., Casey, K., McGahan, E., Gardner, E.A., Rafiee, M. and Blackall, P.J. (2004) Microbiological status of piggery effluent from 13 piggeries in the south east Queensland region of Australia. Journal of Applied Microbiology 97, 883-891.
[7] Cote, C., Masse, D.I. and Quessy, S. (2006) Reduction of indicator and pathogenic microorganisms by psychrophilic anaerobic digestion in swine slurries. Bioresource Technology 97, 686-691.
[8] Hill, V.R. and Sobsey, M.D. (2003) Performance of swine waste lagoons for removing Salmonella and enteric microbial indicators. Transactions of the Asae 46, 781-788.
[9] Pourcher, A.M., Marti, R., Thorigné, A., Jégou, B. and Dabert, P. (2007) Effect of anaerobic storage and aerobic digestion on micro-organisms in pig manure : cultural and molecular approaches. In: Proceedings XIII International Congress in Animal Hygiene ISAH
Vol. 1, Estonian University of Life SciencesTartu, Estonia, June 17-21, 2007, Tartu.
[10] Vanotti, M.B., Millner, P.D., Hunt, P.G. and Ellison, A.Q. (2005) Removal of pathogen and indicator microorganisms from liquid swine manure in mufti-step biological and chemical treatment. Bioresource Technology 96, 209-214.
[11] Pourcher, A., Aktouche, N., Coté, C., Rousseau, P., Godbout, S. and Martinez, J. (2006) Behaviour of enteric micro-organisms in Canadian and French swine manure treatments. In: Procceedings 12th RAMIRAN International Conference-11-13 sept Vol. Aarhus Danemark.
[12] Deportes, I., Benoit-Guyod, J.L., Zmirou, D. and Bouvier, M.C. (1998) Microbial disinfection capacity of municipal solid waste (MSW) composting. Journal of Applied Microbiology 85, 238-246.
[13] Gantzer, C., Gaspard, P., Galvez, L., Huyard, A., Dumouthier, N. and Schwartzbrod, J. (2001) Monitoring of bacterial and parasitological contamination during various treatment of sludge. Water Research 35, 3763-3770.
[14] Tiquia, S.M., Tam, N.F.Y. and Hodgkiss, I.J. (1998) Salmonella elimination during composting of spent pig litter. Bioresource Technology 63, 193-196.
[15] Pourcher, A.M., Morand, P., Picard-Bonnaud, F., Billaudel, S., Monpoeho, S., Federighi, M., Ferre, V. and Moguedet, G. (2005) Decrease of enteric micro-organisms from rural sewage sludge during their composting in straw mixture. Journal of Applied Microbiology 99, 528-539.
[16] Mohaibes, M. and Heinonen-Tanski, H. (2004) Aerobic thermophilic treatment of farm slurry and food wastes. Bioresource Technology 95, 245-254.
[17] Pereira-Neto, T.J., Stentiford, E.I. and Smith, D.V. (1986) Survival of faecal indicator micro-organisms in refuse/ sludge composting using aerated static pile system. Waste Management and Research 4, 397-406.