Investigating helminth eggs and *Salmonella sp.* in stabilization ponds treating septage

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Abstract Sludge management arises as a relevant problem after being accumulated in primary ponds of septage treatment plants. One of the most attractive options for sludge disposal is its use in agriculture and then specific guidelines regarding hygienic quality must be fulfilled. This study aimed at evaluating the storage time needed to inactivate *Ascaris* eggs and *Salmonella* in sludge accumulated in a primary pond treating septage. Raw septage exhibited very low concentrations of viable *Ascaris* eggs, thus experiments with *Ascaris suum* eggs spiking were conducted. The concentration of *Ascaris* eggs in the solids accumulated at the bottom of the pond was 20 eggs/g of total solids (g TS) at the time of pond closure. Although it decreased, some eggs remained viable (0.59 mean viable eggs/g TS) up to 20 months of in-pond storage of the biosolids. *Salmonella* survival was studied after developing an analytical method that inhibited the native flora. Sludge was seeded with *Salmonella enteritidis*. An equation adequately describing *Salmonella* die-off in biosolids subjected to 115 days of in-pond storage/dewatering, was found to be represented by the regression: $y = \log \text{MPN Salmonella/g TS} = 6.67 \cdot t^{-0.086}$, with $t =$ storage time elapsed in days. The initial concentration was 7.0×10^6 MPN/g TS and the removal efficiency was 99 %.

Keywords *Ascaris*; biosolids; helminth eggs inactivation; primary ponds; *Salmonella*; septage

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Introduction

In Argentina, 89% of the 37,000,000 inhabitants are living in small and medium-size towns and in cities. 54% of the urban population are served by sewerage and the remaining 46% are using on-site sanitation systems, mostly septic tanks with soak pits. This paper reports investigations on helminth eggs and *Salmonella* inactivation in-situ and at ambient temperature in biosolids generated during pond treatment of septage (the pump-outs from septic tanks). The use of biosolids in agriculture appears as the most attractive solution as it contributes to recycling organic matter and nutrients. Hence, guidelines or standards on the hygienic quality of the solids must be satisfied. There exists, to date, insufficient published information about the survival of excreted pathogens in the biosolids derived from faecal sludge treatment. Argentina enacted guidelines for the use and disposal of biosolids by adopting US standards (Ministerio de Desarrollo Social y Medio Ambiente 2001; USEPA 1993). They stipulate <1 viable helminth egg/4 g TS and <3 MPN *Salmonella*/4 g TS. Xanthoulis and Strauss (1991) proposed a guideline value for biosolids (as produced in faecal sludge or in wastewater treatment schemes) of 3–8 viable nematode eggs/g TS. This recommendation is derived from the WHO guideline of ≤ 1 nematode egg/litre of treated wastewater used for vegetable irrigation (WHO, 1989), and based on an average manuring rate of 2–3 tons TS/ha year.

Ascaris lumbricoides eggs are particularly important as indicator of the hygienic quality of biosolids as Ascariasis is one of the most widespread excreta-related infections in low-income areas and as *A*. eggs are the most resistant among the gastro-intestinal

pathogens. Their removal suggests that all other pathogens have also been inactivated (Feachem *et al.*, 1983).

Standardized methods for the isolation of *Salmonella* in excreta-derived sludges have not been available to date. Although a proven methodology does exist and is widely applied for blood, food, drinking water, and wastewater samples, it does not work likewise for isolation in biosolids. The reason lies in the co-existence of a native flora which masks *Salmonella* development, particularly if *S.* occur in low concentrations. This lead the authors in previous investigations to develop a methodology, which is suitable to detect *Salmonella* in sludges.

The aims of this study were: 1) To evaluate the required storage time for *Ascaris* eggs inactivation in accumulated sludge of a primary septage pond. 2) To evaluate the die-off of *Salmonella* in the same type of sludge.

The study comprised the following:

(i) Determining helminth eggs viability in biosolids stored and subjected to natural dewatering in-situ in a primary septage pond.
(ii) Determining the viability of *Ascaris suum* eggs spiked into septage pond biosolids which were subjected to natural dewatering/drying in a container and to further drying in an experimental drying bed.
(iii) Determining the *Salmonella* die-off in septage sludge.

The septage-cum-wastewater co-treatment scheme of the town of Alcorta (Province of Santa Fe, Argentina), which was used to conduct the in-situ experiments, is depicted in Figure 1. The town has a population of 5,000 inhabitants, of which 60% are served by septic tanks and 40% by sewerage. The system treats 25 m^3/day of septage and 200 m^3/day of wastewater (Ingallinella *et al.*, 2000; 2002).

Materials and methods

Method for detecting and enumerating helminth eggs and for determining eggs viability

The method used was the USEPA protocol (1992) as modified by Schwartzbrod (1998). The test uses zinc sulfate ($\rho = 1.3$) instead of magnesium sulfate ($\rho = 1.2$) for the flotation. Helminth eggs counts were made in a Sedgwick-Rafter counting chamber with an Olympus Bmax40 Microscope, at 100 × and 200 × magnification. The eggs were differentiated by genus. Egg viability was determined by incubating the processed samples at 20 °C during 4 weeks in H_2SO_4 0.1 N. Viable eggs were those that had a larva inside after incubation. Total and volatile solids were additional parameters analyzed using *Standard Methods* (19th edition, 1995). Rainfall and air temperature were recorded.

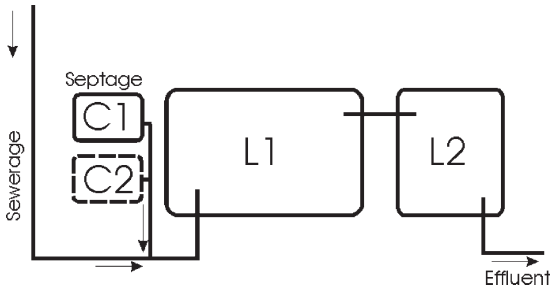

Figure 1 Alcorta (Argentina) pond scheme. C1 + C2 = alternatingly-operated septage sedimentation/digestion ponds; L1 + L2 = facultative and maturation ponds co-treating septage supernatant and raw wastewater

Determining helminth eggs viability in biosolids stored and subjected to natural dewatering in-situ in a primary septage pond (C1)

Sludge of the primary septage pond C1 was allowed to accumulate during the 12-month septage loading period. After this, the supernatant was pumped into the parallel pond C2, which was subsequently receiving the septage.The in-situ *Ascaris* eggs concentration at the beginning of the observation period amounted to 3 *Asc.* eggs/g TS. 42 composite samples of biosolids accumulated in pond C1 were taken over a period of 605 days. Each sample was composited from 6 sub-samples taken from different sites at a depth of 20 cm. The composite samples were collected in 6-L plastic recipients and stored at 4 °C. Sludge samples were taken every two weeks during 20 months.

Determining viability of *Ascaris suum* eggs spiked into septage pond biosolids which were subjected to natural dewatering/drying in a 70-L container (experiment IIA) and to further drying in an experimental drying bed (experiment IIB)

Container experiment (experiment IIA). The *Ascaris suum* eggs spiking experiment was conducted following the finding that helminth egg concentrations in the raw septage and in the accumulated biosolids were too low to conduct inactivation studies in a meaningful manner. *Ascaris suum* eggs were used because they are as resistant as those of *A. lumbricoides* (Carlander and Westrell, 1999), and as they have the advantage of being more easily obtained and less dangerous for humans.

Suspensions of *A. suum* eggs were prepared by removing the uterus of adult female worms, which were collected from the intestines of infected pigs in a slaughterhouse. The last centimetres of the uterus near the vulva containing the mature eggs were cut off. The uterus pieces were put in a test tube to which 15 ml of tap water were added. The uterus pieces were then squeezed with a glass stick to release the eggs. The suspension was then passed through a sieve of 100 μm into another test tube in order to get rid of large fragments. The suspension was concentrated through centrifugation for 10 minutes and the supernatant was withdrawn. In a drop of the pellet, a microscopic count (200 × magnification) of *Ascaris* eggs was made. Five drops of 0.05 ml were counted and the mean concentration of eggs was calculated. This result was extrapolated to the total volume of the pellet. Then the pellet was resuspended in a volume of physiological solution to have a final concentration of approx. 3×10^5 eggs/l.

A test tube with 10 ml *Ascaris* suspension was incubated at 20 °C in order to know whether *Ascaris* eggs in the suspension were potentially fertile. Microscopic observations were made twice a week during 4 weeks.

The container experiment. A 70-L plastic tank was set into the sludge accumulated in pond C1. A portion of sludge and a portion of *A. suum* egg suspension were added to the container simultaneously and homogenised with a stick. New portions of sludge and suspension were added and well mixed until the tank was full. The final concentration in the sludge was 20 *Ascaris* eggs/g TS. The sludge remained in the tank during 8 months, reaching a TS content of 55%. Samples of 400 g were collected at 20 cm depth every 15 days. They were refrigerated at 4 °C prior to analysis.

Viability of Ascaris suum eggs under drying bed conditions (experiment IIB). A plastic box of 40 × 50 × 20 cm size with bottom drainage was used to simulate a drying bed. The sludge, which had previously been stored in an open container for 8 months, was subjected to further drying for 12 months. The sampling was similar to that for the container, yet the size of the composite sample was restricted to 100 g.

Die-off of Salmonella in septage sludge

A suitable methodology for *Salmonella* isolation was investigated in preceding experiments. The respective steps comprised: 1) Enrichment in Rappaport–Vassiliadis broth at 43 °C, 48 hours. 2) Isolation in XLD Agar at 40 °C, 24 hours. A 10-L container filled with solids accumulated in the septage pond was seeded with *Salmonella enteritidis* to study the die-off of *Salmonella*. The container was exposed to environmental conditions during 4 months.

Preparation of Salmonella suspension. Colonies from a pure culture of 24 hours were picked up and suspended in 1 litre of sterile peptoned water. The suspension was then incubated for 24 hours at 35 °C. After centrifugation at 3,500 rpm, the pellet was washed several times with sterile saline solution. After each washing, the suspension was centrifuged and the supernatant was discarded. The pellet was suspended in a sterile saline solution to yield 100 ml. The concentration of *Salmonella* in the suspension was determined by seeding 1 ml of several dilutions in nutrient agar plate by triplicate. The final concentration of the suspension was 7.0×10^9 CFU/ml. When preparing the experimental container experiment, portions of 1 litre of sludge were blended with 1 ml of the suspension of *Salmonella enteritidis* during 1 minute and then poured into the container until a volume of 10 litres was attained.

Sampling. Samples were collected every 3 days during the first 15 days and then once a week during 4 months. pH, total and volatile solids, and faecal coliforms were also analyzed.

Sample preparation for Salmonella detection. 50 g of wet sludge were blended with 500 ml of sterile saline solution + Tween 80 to a 0.1%v/v concentration, during 2 minutes (Method 1682, EPA-821-R-98-004 (USEPA, 1998)).

Isolation, identification and quantification of Salmonella. Samples were analyzed quantitatively (MPN/gTS) applying the method developed by the authors: 10 ml were inoculated in 5 tubes containing 10 ml of double concentration Rappaport–Vassiliadis (RV) broth, 1 ml in 5 tubes with 10 ml of simple broth, 0.1 ml in 5 tubes with 10 ml of simple broth and so on with series of higher dilutions. Control tests inoculating *Salmonella enteritidis* suspension in 5 tubes were done in parallel. The inoculated tubes were incubated at 43 °C for 48 hours. Positive tubes (turbidity) were streaked in XLD agar plates and they were incubated at 40 °C for 24 hours. Typical clear, pink-edged, black-centred *Salmonella* colonies were typified in TSI (alkaline/acid), LIA (positive) and Urease tests (negative). Then XLD plates with *Salmonella* colonies were correlated with the series of positives RV tubes in three significant dilutions (choosing the highest dilution that gives positive results in all 5 tubes), and according to 9221.IV. tables from *Standard Methods*, (19thedition) 1995, the most probable number were determined. Therefore, *Salmonella* MPN/gTS = MPN index/100 ml × 10 divided by the largest significant dilution volume × % TS (EPA/625/R-92/013 (USEPA, 1999)).

Results and discussion

Determining helminth eggs viability in biosolids stored and subjected to natural dewatering in-situ in a primary septage pond (C1)

The concentrations of total helminth eggs over the 22 months observation period varied considerably and ranged from 0.1 to 17 eggs/g TS. *Ascaris* eggs were prevalent and their concentrations varied between 0.1 and 16 eggs/g TS. *Trichuris* and *Hymenolepis* eggs

were also detected. While the A. eggs concentration varied between 2.6 and 16 eggs/g TS during the first 330 days of in-pond storage, its concentration ranged from 0.1 to 1.4 eggs/g TS only between day 346 and 633, except for two samples with higher concentrations (Figure 2a).

The humidity remained constant at 80% during the first 535 days of storage, but gradually decreased to 53% during the subsequent 70 days. The biosolids were removed from the pond and arranged into open piles on day 636. There, the dehydration process continued. Pile samples collected from the piles on day 662 and day 697 exhibited humidities of 39% and 32%, respectively (Figure 2b). Viable helminth eggs were also detected.

Based on the results for the entire observation period of 697 days, there is no apparent direct relationship between sludge humidity and *Ascaris* eggs count. The decrease in eggs concentration from day 330 appears to be related more to storage duration rather than to decreasing humidity, since the humidity started to decrease around day 535 only (Figure 2). Volatile solids (VS) remained approximately constant at 38% of TS during the entire period of observation.

It must be noted that helminth eggs in the primary pond sludge became accumulated during 12 months prior to the beginning of this study. This may have contributed to the weakening of the external membranes of the eggs, which might had been degraded enzymatically by bacteria and fungi present in the sludge.

In the 45 samples analysed, the number of viable helminth eggs was lower than that stipulated by the Argentinian regulations (<1 viable egg/4 g TS). Hence, in this case, helminth eggs cannot serve as a reliable indicator to determine the storage period required to reach a satisfactory hygienic quality. This was the reason for continuing the investigations using artificial additions of *Ascaris* eggs. At the beginning of this experiment, 6 samples were extracted and analyzed in search of *Salmonella*, but all of them were negative.

Viability of *Ascaris suum* eggs spiked into septage sludge stored in a container

Sludge from primary pond spiked with *Ascaris suum* eggs was set into a plastic tank. The tank was located inside this pond and it remained here for almost 8 months. Then the sludge was transferred to a "drying bed" where it remained for the following 12 months.

The concentration of *Ascaris* eggs in the tank was 19 eggs/g TS at the beginning of the experiment. The *Ascaris* eggs concentration varied between 1.8 and 24 eggs/g TS during 213 days (7 months) of storage. This fluctuation might be attributable to a random distribution of the eggs in the sludge, which may have been caused by inadequate mixing during the filling and *A. suum* eggs admixing.

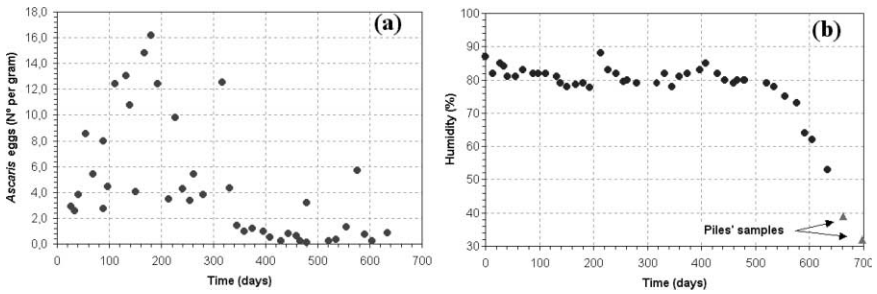

Figure 2 *Ascaris* egg concentration (a) and humidity (b) in pond C1 biosolids vs. storage time

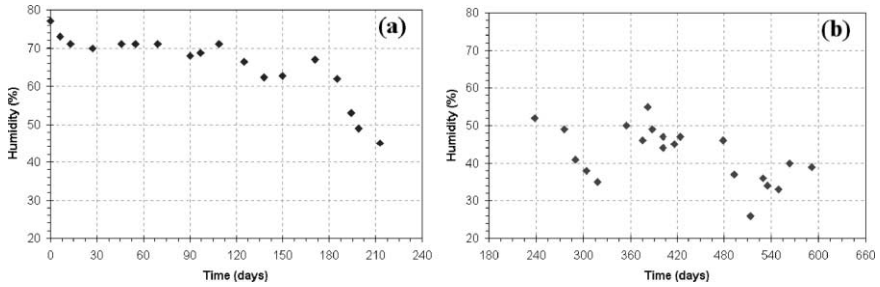

Figure 3 Humidity versus time in container experiment (a) and in drying bed (b)

Humidity ranged from 68% to 77% during the first 120 days (Figure 3a), while the *A. suum* concentration varied between 5.4 and 24 eggs/g TS (Figure 4a). No apparent relation between humidity and eggs concentration could be established during this period since the humidity remained constant. The eggs concentration decreased significantly from15 to 1.8 *Ascaris* eggs/g TS max. and min., respectively between days 120 and 213. The humidity decreased from 67% to 45% simultaneously.

Viable eggs ranged from 0 to 5.4 viable eggs/g TS and the percentage of viability (ratio of viable vs. total eggs) was between 0% and 45%. However, higher percentages did not correspond to higher concentrations. Figure 4a shows that the total egg concentration varied considerably during the experimental period while the concentration of viable eggs remained almost constant at a relatively low level.

As in the in-situ experiment, the volatile solids (VS) content remained fairly constant at 29%. No correlation between VS and *A. suum* egg survival could be detected.

Viability of *Ascaris suum* under drying bed conditions

The sludges were moved to an experimental "drying bed" after 8 months of dewatering/drying in the experimental container (experiment IIA), The initial *Ascaris* egg concentration was of 13 eggs/g TS. The sludge remained on the drying bed during 12 months (Jan. – Dec. 2003). *Ascaris* egg concentrations ranged between 0.4 and 4.4 eggs/g TS during this period and were thus lower than in the preceding container experiment. The humidity varied between 55% and 26% during the 353 days of the drying phase (Figure 3b).

Figure 4b illustrates the gradual decrease of *Ascaris* eggs concentration. Observed egg viability fluctuated between 0.2 and 2.3 viable *Ascaris* eggs/g TS and the percentage of viable eggs ranged from 32% to 81%. There was no correlation between concentration and percentage, as low concentrations of viable eggs partly coincided with high percentages of viability. Even though the concentration of *Ascaris* eggs dropped in the drying bed relative to that obtained in the tank, the concentration of viable eggs did not decrease in the same proportion (Figs. 4a and b). This indicates that the viable eggs were more resistant than non-viable ones under the same adverse environmental conditions (Johnson

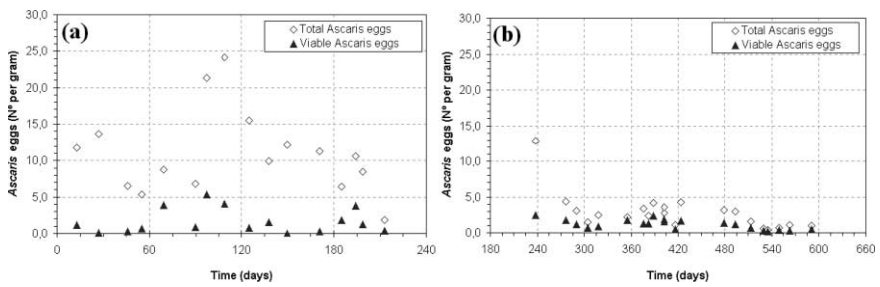

Figure 4 *Ascaris* concentrations vs. time: (a) container experiment (b) drying bed

et al., 1998). Hence, the *Ascaris* eggs that had disappeared could be those that did not have the capacity of developing into larvae because they were less evolved.

The humidity was amounted to 45% at the onset of the drying bed experiment and decreased to approx. 26% after 240 days. Figure 5 shows that the higher viability corresponded rather well with humidity values above 40%. At the end of the experiment, the concentration of viable eggs decreased to 0.2–0.4 viable *Ascaris* eggs/g TS while humidity decreased to below 40%.

A statistical study (ANOVA) was carried out in order to evaluate the relation of viability of eggs with humidity and time. The analysis shows that there was a statistically significant difference (at 95% confidence level) between the mean viable *Ascaris* eggs concentration (1.4 eggs/gTS) from one level of humidity range (40–55%) to another level (26–40%) with a mean viable eggs concentration of 0.59 eggs/g TS. The same statistical analysis showed that time is not an influential factor for viable *Ascaris* eggs counts in the same way as is humidity under these experimental conditions. Viable eggs concentrations, even though they exhibited highly variable values, decreased in parallel to a decrease of humidity to values in the range 26–40% along the whole drying period of 500 days (tank and drying bed).

Die-off of *Salmonella* in septage sludge

To minimize native flora development, the following method for *Salmonella* isolation technique was found most suitable after assaying different enrichment broths and isolation agars at different incubation times and temperatures: 1) Enrichment in Rappaport-Vassiliadis broth at 43 °C, 48 hours. 2) Isolation in XLD Agar at 40 °C, 24 hours. Identification of suspected colonies by biochemical tests: TSI, LIA, Urease and serological confirmation with Group O Antigen. To study the survival of *Salmonella* in the sludge, an experimental container with sludge seeded with *Salmonella enteritidis* was used. The proposed methodology mentioned above for *Salmonella* isolation was applied and the multiple test tubes method used for quantification. The results obtained during 4 months of experience are shown in Table 1.

The concentration of *Salmonella* was 7.0×10^6 MPN/g TS at the beginning of the experiment. The concentration decreased to 1.7×10^5 MPN/gTS within 6 days, remaining at a geometric mean of 1.4×10^5 MPN/gTS for the next 30 days. It can be remarked that the concentration increased in one log unit at approx. 50 days of storage possibly due to abundant rains during the previous days, which created an optimum humidity level for *S.* multiplication (Sidhu *et al.*, 2001). A similar behaviour was detected at 100 days of storage (Figure 6). The concentration of *Salmonella* decreased to a mean value of

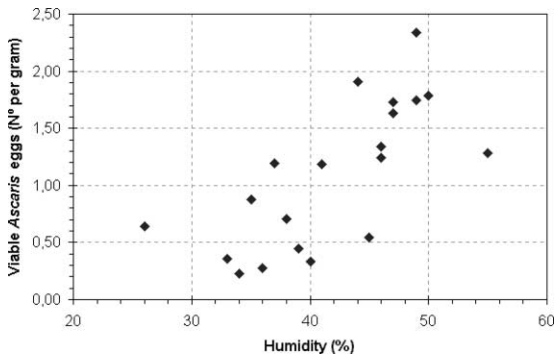

Figure 5 Ascaris viability versus humidity in drying bed

Table 1 Die-off of *Salmonella sp.* in sludge in experimental container

Days	pH	Hum %	TS %	FS %	VS %	F. Coliforms (MPN/gTS)	*Salmonella* (MPN/gTS)	Ambient Temp. (°C)	Sludge Temp. (°C)
1	7.3	71	29	78	22	3.1×10^4	7.0×10^6	15.0	14.5
3	6.9	71	29	75	25	1.7×10^4	2.1×10^6	20.0	20.0
6	7.3	70	30	75	25	1.1×10^4	1.7×10^5	13.0	15.0
10	7.5	68	32	75	25	2.1×10^4	3.4×10^5	14.0	14.5
16	7.5	63	37	78	22	1.2×10^4	1.8×10^5	20.0	19.0
23	7.5	63	37	78	22	3.5×10^3	1.3×10^5	15.0	17.0
30	7.5	52	48	77	23	7.9×10^2	2.8×10^4	20.0	18.0
37	7.2	39	61	77	23	1.0×10^3	2.0×10^5	28.0	26.0
51	6.5	38	62	78	22	1.2×10^4	1.9×10^6	28.0	29.5
65	6.5	27	73	79	21	1.6×10^3	5.6×10^4	27.0	26.5
79	6.5	20	80	79	21	1.8×10^3	4.2×10^4	26.0	26.5
100	7.0	47	53	80	20	7.5×10^2	3.2×10^5	23.0	25.0
115	7.5	44	56	82	18	3.8×10^2	3.0×10^4	27.0	29.0

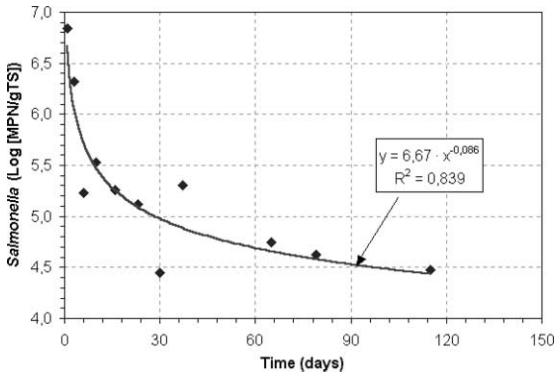

Figure 6 Salmonella die-off in biosolids

3.8×10^4 MPN/g TS after 65 days. In summary, the decay after 115 days of storage was 2 log units (from 10^6 to 10^4 MPN/g TS).

Humidity decreased from 71% to 20% during the first 79 days of the experiment while the *Salmonella* concentration decreased 2 log units during the same period.The concentration of faecal coliforms in the biosolids was 3.1×10^4 MPN/gTS initially. It decreased to 3.8×10^2 MPN/g TS by the end of the experiment. Note needs to be taken that faecal coliforms were already in the stored sludge while *Salmonella* were seeded at high concentrations at the onset of the experiment.

Conclusions and recommendations

Ascaris eggs present in septage sludge stored and subjected to dewatering during 20 months remained viable, even with humidity as low as 26%.

The viability of *Ascaris* eggs in stored septage sludge decreased when the humidity dropped to below 40%.

Salmonella die-off in biosolids stored over a period of 115 days, can be expressed by the equation: $y = \log \text{MPN Salmonella/g TS} = 6.67 \cdot t^{-0.086}$, where t is the elapsed time in days. The removal efficiency was 99%, with an initial concentration of 7.0×10^6 MPN/g TS.

A lower initial concentration of *Salmonella* ($1 \times 10^2 - 1 \times 10^3$MPN/gTS) is recommended to be seeded in sludge to verify whether the survival curve matches with the one obtained at higher concentrations.

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