

## Isolation of *Salmonella* sp. in sludge from septage treatment plant

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**Abstract** Waste stabilization ponds (WSP) are an often-used option to treat faecal sludges collected from on-site sanitation systems. Since agricultural use is one of the most attractive options for sludge disposal, specific guidelines on the hygienic sludge quality must be fulfilled, such as for viable helminth eggs and *Salmonella* sp. Although *Salmonella* isolation methods are well known for other types of samples, they are not suitable for faecal sludge. The reason can be attributed to the co-existence of a native bacterial sludge flora masking *Salmonella* development, especially if this bacteria is present at low concentrations. In order to select the best methodology for *Salmonella* recovery from septage sludge, different culture media were assayed at different incubation periods and temperatures. The proposed methodology for *Salmonella* recovery from sludge can be summarised as follows: (1) enrichment in Rappaport-Vassiliadis broth at 43°C, 48 hours, and (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.

**Keywords** *Salmonella* isolation technique; septage; sludge

### Introduction

Waste stabilization ponds (WSP) are an often-used option to treat faecal sludges collected from on-site sanitation systems. The Sanitary Engineering Center of the University of Rosario, Argentina, in collaboration with the Swiss Federal Institute for Environmental Science and Technology, Switzerland, has been investigating septage treatment in ponds since 1998 (Ingallinella *et al.*, 2002). Research is currently focusing on the inactivation of pathogens. Since agricultural use is one of the most attractive options for sludge disposal, specific guidelines on the hygienic sludge quality must be fulfilled, such as for viable helminth eggs and *Salmonella* sp. Although information on pathogen inactivation in fresh sludge is available, information on pathogen survival in sludges produced during septage treatment is scarce. Therefore, the effect of natural dewatering/drying under temperate climatic conditions on the hygienic quality of accumulated sludges was investigated.

*What microbiological criteria are required for biosolids to be used in agriculture?* Argentinian Regulations on the use and disposal of biosolids (Ministerio de Desarrollo Social y Medio Ambiente, 2001) stipulate < 3 MPN / 4 g Total Solid (TS) for *Salmonella* and < 1 viable egg / 4 g TS for helminth eggs, thereby following USEPA standards (USEPA, 1993).

*What are the difficulties encountered at the laboratory for Salmonella detection?* Although *Salmonella* isolation methods are well known and widely applied in practice for blood, food, drinking water, and wastewater samples, they do not work likewise for faecal sludge. The reason can be attributed to the co-existence of a native bacterial sludge flora masking *Salmonella* development, especially if this bacteria is present at low concentrations.

The guidelines propose  $<3$  MPN/4gTS. Does this value mean that *Salmonella* is actually lower than this concentration, or may it be present at higher concentrations and is unable to be detected due to the abundant competitive indigenous flora existing in faecal sludge? Consequently, a methodology must be developed which can be applied on sludge accumulated in WSP treating septage.

This study aimed at developing a suitable method to isolate and identify *Salmonella* sp. in faecal sludge containing a high concentration of competitive indigenous flora. The investigations were conducted with the sludge from a primary pond of the Alcorta treatment plant, Province of Santa Fe, Argentina (Ingallinella et al., 2002).

### Material and methods

Before investigating a suitable method for *Salmonella* isolation, sludge samples from a primary pond were analysed applying *Standard Methods* (20<sup>th</sup> edition, 1999) techniques. As *Salmonella* was not detected in any of the samples collected over a three-month period, other tests, such as seeding sludge with *Salmonella enteritidis*, were carried out to find an appropriate methodology.

#### Application of the Standard Methods technique

Seven sludge samples from a primary pond treating septage were collected during three months for *Salmonella* isolation. After appropriate homogenization of the sample, 30 g of sludge were blended with 270 ml of sterile physiological solution for one minute. The sample preparation steps recommended in EPA 625/R-92/013-Appendix F were used to determine *Salmonella* (USEPA, 1999). The processed samples were analyzed according to the *Standard Methods* (20<sup>th</sup> edition, 1999). The samples were seeded in the enrichment broth Selenite-Cystine (Merck) and positive cultures were then streaked for isolation in selective Xilose-Lysine-Desoxycholate agar (XLD, Merck). Suspected *Salmonella* colonies were picked to the following biochemical tests: Triple Sugar Iron (TSI), Lysine Iron Agar (LIA) and urease as well as serologically confirmed using the Antigen "O" Group. Additional parameters analysed comprised faecal coliforms; total (TS), fixed (FS), and volatile (VS) solids. The parameters were analyzed according to the *Standard Methods* (19<sup>th</sup> edition, 1995). Rainfall and ambient temperature were recorded during the experiment.

#### Application of the modified technique

Since the preliminary experiment (7 sludge samples) revealed a total absence of *Salmonella* in all samples, development of the influence of the indigenous bacteria masking *Salmonella* development could not be neglected. *Salmonella* is known to be strongly inhibited by native flora present in sludge (Sidhu et al., 2001). It was therefore necessary to investigate another methodology which could better inhibit the competitive bacteria. Therefore, five different sludge samples were seeded with a suspension of *Salmonella enteritidis* (McFarland 0.5). A final concentration equivalent to MPN/g TS  $1.5 \times 10^3 - 1.5 \times 10^5$  was obtained after blending. The seeded samples were processed according to EPA 625/R-92/013-Appendix F for qualitative analysis. In order to select the best methodology for *Salmonella* recovery, different culture media proposed by USEPA (1998), *Standard Methods* (20<sup>th</sup> edition, 1999) and other authors (Gibbs et al., 1997) were assayed at different incubation periods and temperatures.

Enrichment broths:

- (1) Selenite-Cystine broth (Merck) in 24, 48 and 72 hours at 41°C
- (2) Rappaport-Vassiliadis (Merck) in 24,48 and 72 hours at 43 °C

Isolation agars:

- (1) XLD agar (Merck) in 24 hours at 35°C and 40°C
- (2) SS (Salmonella-Shigella) agar (Merck) in 24 hours at 35°C and 40°C

Suspected colonies of *Salmonella* were typified in TSI, LIA and in a urease test. The positive colonies were confirmed by serological tests conducted by the Service of Enterobacteria of the Dr. Carlos Malbrán Institute (<http://www.anlis.gov.ar/>), Buenos Aires, Argentina.

## Results and discussion

### Application of the Standard Methods technique

Seven sludge samples from a primary pond treating septage were analysed. The results are given in Table 1.

### Application of the modified technique

Based on the results obtained (absence) pertaining to the isolation of *Salmonella* in sludge and the significant development of indigenous flora in the cultures, it was necessary to develop a more accurate methodology to ensure *Salmonella* recovery. *Salmonella enteritidis* were seeded in five different sludge samples as previously described. The results obtained by triplicate tests are shown in Tables 2 and 3.

The abundant development of *Salmonella* at 43°C during 48 hours revealed that Rappaport-Vassiliadis was the best enrichment broth. When comparing the two isolation media, SS agar and XLD agar, better results in *Salmonella* recovery were attained with XLD at 40°C during 24 hours of incubation, as the sludge native flora was greatly inhibited.

The proposed methodology for *Salmonella* recovery from septage sludge can be summarised as follows: first, enrichment in Rappaport-Vassiliadis broth at 43°C, 48 hours;

**Table 1** Physical and microbiological parameters of sludge accumulated in a primary pond treating septage

Sample	<i>Salmonella</i> sp (presence/absence)	Faecal coliforms (MPN/100 ml)	Faecal coliforms (MPN/g TS)	Humidity %	TS %	FS %	VS %
1	Absence	504	280	82	18	63	37
2	Absence	165	110	85	15	60	40
3	Absence	120	75	84	16	60	40
4	Absence	60	32	81	19	64	36
5	Absence	160	84	81	19	63	37
6	Absence	160	94	83	17	60	40
7	Absence	140	78	82	18	60	40

**Table 2** Selection of enrichment broth and incubation time for *Salmonella* development

Sample	Selenite-Cistine broth (41°C)						Rappaport-Vassiliadis broth (43°C)					
	<i>Salmonella</i>			Indigenous flora			<i>Salmonella</i>			Indigenous flora		
	24 hs.	48 hs.	72 hs.	24 hs.	48 hs.	72 hs.	24 hs.	48 hs.	72 hs.	24 hs.	48 hs.	72 hs.
1	**	**	*	*	**	**	**	***	**	*	*	**
2	**	*	*	*	**	***	**	***	**	*	*	*
3	*	*	*	*	**	***	*	**	**	*	*	*
4	**	*	*	*	**	**	*	**	**	*	*	**
5	**	**	*	*	**	**	**	***	**	*	**	**

\*Poor development \*\* Moderate development \*\*\* Abundant development

**Table 3** Selection of isolation media and incubation temperature for *Salmonella* colonial growth

Sample	SS agar				XLD agar			
	Salmonella		Indigenous flora		Salmonella		Indigenous flora	
	35°C	40°C	35°C	40°C	35°C	40°C	35°C	40°C
1	*	**	**	*	**	***	**	*
2	**	**	**	*	**	***	*	*
3	*	**	**	**	*	**	*	*
4	**	**	**	*	*	**	**	*
5	*	**	**	**	**	***	**	*

\*Poor development \*\*Moderate development \*\*\*Abundant development

secondly, isolation in XLD agar at 40°C, 24 hours, and identification of suspected colonies by biochemical tests: TSI, LIA, urease and serological confirmation with Group O Antigen.

### Conclusions and recommendations

The most appropriate methodology for isolating *Salmonella sp.* in sludges and inhibiting the development of indigenous flora is: enrichment in Rappaport-Vassiliadis broth, 48 hours at 43°C; then isolation in XLD agar, 24 hours at 40°C; and the use of identification tests: TSI, urease, LIA, serological tests.

Further studies are recommended to verify the effectiveness of the technique: first, using very low concentrations of *Salmonella* in sludge (<10<sup>2</sup> MPN/g TS); secondly, enlarging the number of samples to enhance statistical analysis; and finally, testing another inhibitory factor for indigenous flora.

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