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Assessment of the Accuracy of A Method Used for Quantification of *Ascaris* Eggs in Sewage Sludge

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Abstract: The application of sewage sludge on arable and grassland soils is dependent on the assurance that the concentrations of pathogens present in the biosolids do not pose health risks to either individuals or animals. Therefore, the use of appropriate protocols for the detection and quantification of pathogens in sludge samples is essential. An ideal method for the quantification should provide high sensitivity, accuracy and precision, but shall also follow a program that ensures the quality of the data produced. Methodologies that ensure accurate and reliable responses in a short period of time are also desirable. Therefore, this work aimed at the assessment of the accuracy of the method specified by the U.S. Environmental Protection Agency (USEPA) and used for the quantification of viable Ascaris eggs eventually present in biosolids. This study was carried out using samples of anaerobic sewage sludge collected from two drying beds, one receiving sludge from an upflow anaerobic sludge blanket reactor, and the other receiving sludge from a septic tank.

Key words: anaerobic sludge; *Ascaris suum*; helminths; parasitology of sludge, quantification methods.

1. Introduction

Sewage sludge is the primary waste generated from the treatment of domestic wastewater. Due to its diverse chemical composition and high pathogen load a proper disposal has to be designed [1-3]. It has also to be considered that its agricultural use should not impose a decrease in the quality of soil, surface and groundwater. More importantly, it shall not pose any risk of either biological or chemical nature that could affect the health of living beings or the environment [4]. Therefore, the pathogens that might be present in sewage sludge samples have to be considered as the main issue in any monitoring plan. The pathogens can be enteric viruses, such as adenoviruses and enteroviruses; thermotolerant bacteria that are of fecal origin, such as *Escherichia coli* and *Salmonella*; as well as helminths in the form of viable eggs of *Ascaris*, which can become adults under appropriate medium conditions [2, 5, 6].

Although many pathogens are found in sludge samples, their presence does not necessarily indicate that there is a risk of contamination [7]. This risk depends on the infectious dose that is required for that organism to become pathogenic in an individual [8]. For instance, the minimum infective dose for helminths is very low, and the contact with or the ingestion of a single viable egg can lead to the development of a parasite-associated disease [9]. Therefore, the low infectious dose associated with the resistant structure of helminth eggs makes this parasite the primary target of sewage sludge cleaning techniques.

Hence, if after any treatment the sludge is to be applied in soils and if the spread of these pathogens in the environment is to be prevented, regulatory agencies in several countries have established limits for the concentration of helminth eggs in sewage sludge [5, 10-14]. However, there is no general agreement among researchers, environmental agencies and other government agencies in relation to the most

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appropriate method to be used in the determination of the number of viable eggs in sludge samples [15-18]. Thus, in order to produce more reliable results regarding the identification and quantification of viable helminth eggs, the methodologies currently in use are in need of reassessment.

Among all methodologies used to quantify viable helminth eggs, the USEPA protocol has been widely used. However, this protocol is both a painstaking sample preparation and a meticulous process of examination. For instance, the flotation step, that follows the desorption of the eggs, is the most difficult due to the adjustment of the density of the flotation solution of magnesium sulfate ($MgSO_4$), which is recommended by the protocol.

In the USEPA protocol it can be read that the specific gravity of the solution of magnesium sulfate must be kept at 1.20. Hence, in order to prevent a loss of eggs during this stage of the procedure, and considering that the specific gravity of the *Ascaris* eggs is 1.129 [19], the density of the solution should be checked and adjusted if necessary. Furthermore, due to the interference caused by the presence of cellular debris, yeast cells and also pollen grains, among others, one of the major problems to be faced when the quantification of the eggs is carried out using optical microscopy is related to the requirement of a vast experience and visual acuity of the analyst. Hence, in order to produce robust and reliable results, and also to avoid either over or underestimation of the real number of eggs, the analyst has to receive a thorough training and, in particular, has to be committed to the reliability and accuracy of the results.

Thus, based on the information presented above, the aim of this paper is to present results of tests carried out to assess the accuracy of the quantification of *Ascaris suum* in samples of sewage sludge from two drying beds, using the methodology specified by the United States Environmental Protection Agency [4].

2. Material and Methods

In order to carry out the experiments based on the USEPA protocol, viable eggs of *Ascaris suum* were purchased from Excelsior Sentinel (Ithaca, NY).

Six different samples of sewage sludge were collected from two drying beds which received the sludges that had been previously centrifuged. Three of these samples were collected from a drying bed that contained sludge from an Upflow Anaerobic Sludge Blanket reactor (UASB), and the other three were collected from a drying bed that contained sludge from septic tanks. The two drying beds were located at the sewage treatment plant Fazenda Rio Grande (25°39'31"S, 49°18'32" W), Paraná State, Brazil.

The accuracy of the method specified by the USEPA for the quantification of viable *Ascaris* eggs was assessed by split/spike assays (Fig. 1), in triplicates. The first step was the sterilization of the sludge collected. This was carried out by autoclaving the samples at 121°C and 1.5 kg/cm^2, for 60 minutes. Subsequently, a volume of sewage sludge, equivalent to 50 g of total solids (TS), was disposed in a beaker and inoculated with 100 eggs of *A. suum*. The samples were then incubated at 28ºC, for 21 days. Afterwards, the inoculated eggs recovered were counted using optical microscopy.

According to Bowman *et al*. [20], it is expected that the USEPA method presents an accuracy between 75% and 80%.

3. Results and Discussion

At this point it is worth mentioning that the steps required by the USEPA protocol aim at the desorption of helminth eggs from the sludge matrix. This is achieved by removing lipidic material from the samples, followed by recovering the eggs of *Ascaris* by means of a flotation solution ($MgSO_4$). It is important to stress that the specific gravity of the flotation solution (d = 1.20) allows the recovery of helminth eggs, such as *A. suum, Toxocara canis* (d = 1.090) and *Ancylostoma caninum* (d = 1.056). Nevertheless, the

flotation solution with this specific gravity does not allow the recovery of heavier eggs, such as *Taenia* sp. (d = 1.225) and *Physaloptera* spp. (d = 1.237) [19]. Considering that the USEPA protocol recommends *A. suum* eggs as a standard for the identification and quantification of viable helminth eggs, results produced for the quantification of helminths in general using the procedures specified by this protocol can be biased. This is due to the fact that the method is intended to recover *Ascaris* eggs solely.

In fact, Barés and colleagues [18], in a study aimed at the improvement of a methodology used for the quantification of viable helminths eggs, investigated the use of the solution of zinc sulfide (d = 1.20) for the recovery of viable eggs of *Taenia* sp.. According to the authors, methodologies that utilize flotation solutions with this specific gravity are not effective to retrieve eggs of some species of helminths, and are prone to underestimate the presence of the parasites in the samples. In this case, and in order to ensure the recovery of heavier eggs, the authors recommended the use of flotation solutions with specific gravity of 1.30.

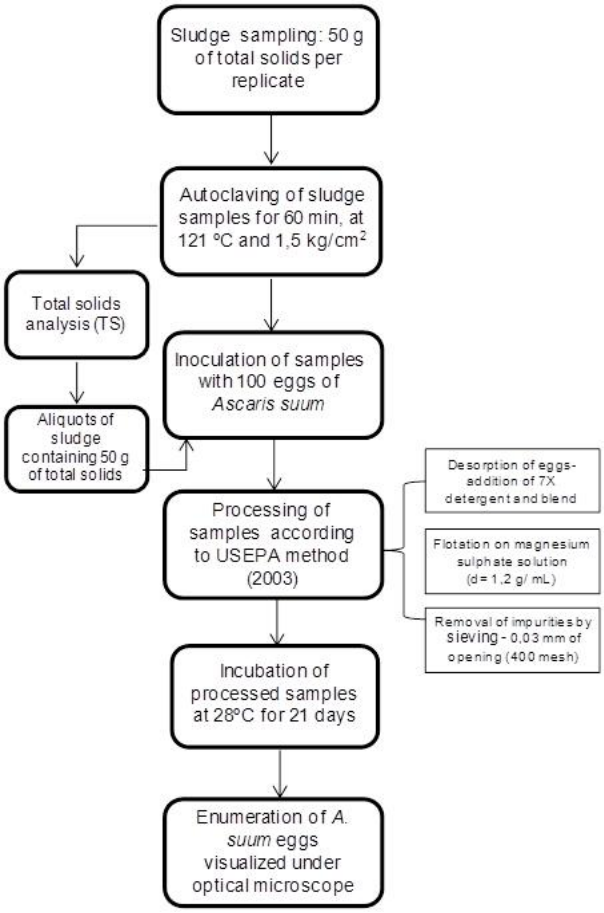

Fig. 1 Schematic representation of the steps of the split/spike assays.

The presence of interferences in the microscopic field during the quantification of *A. suum* eggs was also a major difficulty faced during the development of this research. Fig. 2 presents some interferences observed when optical microscopy was used for the quantification of the eggs recovered. An egg of *Ascaris* was observed among various artifacts that were present in the sample.

Hence, it is highly recommended the removal of all small solids and debris from the samples during the steps of desorption, flotation and sieving. This operation is decisive for the achievement of correct visualization and quantification of viable helminth eggs under optical microscopy.

In the course of the microscopic analysis, it was observed that some of the *A. suum* eggs remained in early embryonic stages, even after the incubation period of 21 days specified by the protocol. Some of these stages can be seen in Fig. 3. However, according to the USEPA protocol, these eggs should be considered nonviable. Nevertheless, as it can be seen in Fig. 3, they remained viable and formed the larva. In

Fig. 2 Interferences on the Visualization of Helminths Using Optical Microscopy.
Note: the arrow indicates an egg of *Ascaris suum*.

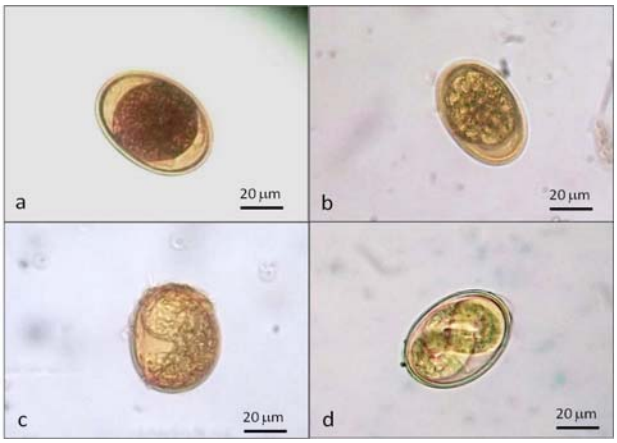

Fig. 3 Different Stages of Development of Eggs of *Ascaris suum* under Optical Microscopy: (a) One Cell Stage; (b) Late Morula; (C) Gastrula; (D) Larval Stage (L2).

fact, other authors [22] referred to periods of two months to complete the development of eggs of *A. suum*. These authors pointed out that the variation of the time for embryogenesis of *A. suum* is due to factors such as the type of the sludge evaluated, as well as the concentration of the parasite eggs in the sludge samples.

Regarding the accuracy obtained for the assays, samples from the drying bed that contained sludge from septic tanks, presented an average recovery of *A. suum* eggs of 74% (±6.1%). This value is in accordance with that expected for the recovery of the eggs, which is between 75% and 80% for anaerobic sludge as verified by Bowman et al. (2003). However, samples collected from the drying bed that contained UASB sludge presented 54% (±4.2) of recovery of the inoculated eggs. One possible cause for the lower value could have been due to interferences when the eggs were being counted using optical microscopy.

The difference in the recovery of *A. suum* eggs for sludge samples that have similar characteristics, that is, both anaerobic and disposed of in drying beds, was unforeseen. In fact, when the accuracy of the USEPA protocol was tested by Bowman and colleagues [21], only four matrices of biosolids were evaluated, namely: (1) synox-treated biosolids (acid treatment process); (2) anaerobically treated biosolids stored in lagoons; (3) soil-biosolid blends; and (4) biosolids from Chemfix process (alkaline treatment). However, the USEPA (2003) generalizes the application of this methodology for samples of sewage sludge and compost (Appendix I EPA/625/R-92/013).

As it was observed from the results produced by this research, the generalization of the USEPA protocol can lead to an inappropriate application of this methodology and, thus, the attainment of inconsistent results.

4. Conclusions

The results produced when dehydrated sludge from a septic tank was used showed an accuracy of 74% for the quantification of viable eggs of *Ascaris suum*, which is accordance with the USEPA protocol. Nonetheless, the method was not effective for samples of dehydrated UASB sludge. In this case, only 54% of the inoculated eggs of *A. suum* were recovered, considerably below spectation, which was to be between 75% and 80%.

During the split/spike assays, some major difficulties could be identified. One that can be cited is the correct adjustment of the solution of magnesium sulfate, for the flotation and adequate recovery of the eggs of *A. suum* from the sludge samples; and the other is the presence of interferences which can be associated with the visualization and quantification of the eggs under optical microscopy, that could have led to inaccurate results.

For most anaerobic sludges, the suitable methodology for the determination and quantification of viable *Ascaris* eggs is that specified by the USEPA protocol. Nevertheless, different matrices of sludge, other than those mentioned before, are being characterized by this protocol. This could result in the underestimation of the presence of eggs of *Ascaris* in the samples.

Therefore, in order to avoid any risk to public health and the environment, it is important to evaluate several matrices of sewage sludge. If this strategy is adopted, the method under discussion could be

validated. It would be an achievement in this field because until now there is no agreement on the most adequate methodology for the quantification of viable helminth eggs.

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References

[1] D. Fytili and A. Zabaniotou, Utilization of sewage sludge in EU application of old and new methods — A review, *Renewable and Sustainable Energy Reviews* 12 (2008) 116-140.

[2] J. P. S. Sidhu and S. G. Toze, *Human pathogens and their indicators in biosolids: A literature review*, *Environmental International* 35 (2009) 187-201.

[3] L. Feng, J. Luo and Y. Chen, Dilemma of Sewage Sludge Treatment and Disposal in China, *Environmental Science & Technology* 49 (8) (2015) 4781-4782.

[4] USEPA-U.S. Environmental Protection Agency, Control of Pathogens and Vector Attraction in Sewage Sludge, EPA 815-R-06-002, Revised Edition, 2003.

[5] USEPA-U.S. Environmental Protection Agency, Control of Pathogens and Vector Attraction in Sewage Sludge, EPA/625/R-92/013, 1992.

[6] T. M. Straub, I. L. Pepper and C. P. Gerba, Hazards from pathogenic microorganisms in land disposed sewage sludge, *Reviews of Environmental Contamination and Toxicology*132 (1993) 55-91.

[7] D. L. Lewis and D. K. Gattie, Pathogen risks from applying sewage sludge to land, *Environmental Science and Technology* 36 (2002) 13.

[8] C. P. Gerba and J. E. Smith, Sources of pathogenic microorganisms and their fate during land application of wastes, *Journal of Environmental Quality* 34 (2005) 42-48.

[9] I. Navarro, B. Jiménez, S. Lucario and E. Cifuentes, Application of helminth ova infection dose curve to estimate the risks associated with biosolids application on soil, *Journal of Water and Health* 7 (2009) 31-44.

[10] Swiss Government, Ordinance on substances dangerous to the environment, *Journal Officiel.* (1992) 16.

[11] European Comission, Disposal and recycling routes for sewage sludge Part 2 – regulatory report, Official Publications of the European Communities, Luxembourg, 2001.

[12] NOM-004-SEMARNAT, *Proteccion ambiental-lodos y biosolidos - especificaciones y limites maximos permisibles de contaminantes para su aprovechamiento y disposicion final "Environmental Protection, Sludge and Biosolids — Specifications and Maximum Contaminant Limits for Use and Final Disposal"*, Mexico: Diario Oficial, 2002, pp. 18-61. (in Spanish)

[13] CONAMA - Conselho Nacional do Meio Ambiente, Define critérios e procedimentos, para o uso agrícola de lodos de esgoto gerados em estações de tratamento de esgoto sanitário e seus produtos derivados, e dá outras providências "Resolution 375. Define criteria and procedures for the agricultural use of sludge produced during sewage treatment and its derived products, and provides other measures". Diário Oficial da República Federativa do Brasil, 2006, pp .141-146. (in Portuguese)

[14] WHO - World Health Organization, Guidelines for the safe use of wastewater, excreta and greywater. Volume IV: Excreta and greywater use in agriculture, World Health Organization Press, Geneva, Switzerland, 2006.

[15] K. L.Nelson and J. L. Darby, Inactivation of viable Ascaris eggs by reagents during enumeration, *Applied and Environmental Microbiology* 67 (2001) 5453-5459.

[16] National Research Council, *Biosolids Applied to Land: Advancing Standards and Practices*, The National Academies Press, Washington, DC., 2002.

[17] Z. Szabo and B. Vargha, Horizontal standards on hygienic parameters for implementation of EU directives on sludge, soil and treated bio-waste, Horizontal-HYG DL 2/3.2 Report, Budapest, 2006, pp. 1-33.

[18] M. E. Barés, M. C. B. Braga, A. Noyola and M. M. Aısse, Improvement of the method for the determination of helminth eggs in sewage sludge, in: *XXXII Congreso Interamericano de Ingeniería Sanitaria y Ambiental*, Punta Cana, Dominican Republic, Vol. 1, 2010, pp. 1-7. (in Portuguese).

[19] E. D. David and W. D. Lindquist, Determination of the specific gravity of certain helminth eggs using sucrose density gradient centrifugation, *The Journal of Parasitology* 68 (5) (1982) 916-919.

[20] J. Pritchard, Ready auto pilot: Google to build prototype of truly driveless car, Waterbury Republican American, May 29, 2014, pp. C-1.

[21] D. D. Bowman, M. D. Little and R. S. Reimers, Precision and accuracy of an assay for detecting Ascaris eggs in various biosolid matricesm *Water Research* 37 (2003) 2063-2072.

[22] M. C. V. Rocha, Application of assays of embryogenesis and RT-qPCR for determination of the viability of Ascaris suum eggs in sewage sludge, thesis, Water Resources and Environmental Engineering Post-Graduate Program, Parana Federal University, Brazil, 2015, p. 172.