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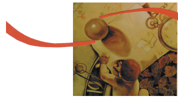

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CIVIL ENGINEERING

Quantification and analysis of the viability of (oo)cysts of pathogenic protozoa in sewage sludge

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ABSTRACT. For the use of sewage sludge, it is extremely important to consider the microbiological aspect of this byproduct that poses direct and indirect risks to public health as for its inadequate handling and use. This study aimed to quantify *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts and infer their viability in sludge samples from two sewage treatment plants. The method used consisted of three successive centrifugations followed by immunofluorescence assay and staining with propidium iodide. Samples were 100% (14/14) positive for cysts and 57.1% (8/14) for oocysts, with higher concentrations and mean viability above 75%. The change in the treatment of activated sludge, from extended aeration to conventional system, caused no difference in the concentration and viability of (oo)cysts.

Keywords: *Cryptosporidium*, *Giardia*, activated sludge, reuse.

Quantificação e análise da viabilidade de (oo)cistos de protozoários patogênicos em lodo de esgoto

RESUMO. Com vista à utilização de subprodutos do tratamento de esgoto sanitário, a questão microbiológica do lodo de esgoto é extremamente importante, pois revela riscos diretos e indiretos à saúde pública quanto a sua manipulação e uso inadequados. Este trabalho buscou quantificar cistos de *Giardia* spp. e oocistos de *Cryptosporidium* spp., bem como inferir a sua viabilidade, em amostras de lodo de duas estações de tratamento de esgoto. Foi utilizado método com três centrifugações sucessivas, seguido de reação de imunofluorescência direta e coloração com iodeto de propídeo. As amostras foram 100% (14/14) positivas para cistos e 57,1% (8/14) para oocistos, com elevadas concentrações e viabilidade média acima de 75%. A mudança do sistema de tratamento de lodos ativados, de aeração prolongada para convencional, não mostrou diferença na concentração e na viabilidade dos (oo)cistos.

Palavras-chave: *Cryptosporidium*, *Giardia*, lodos ativados, reuso.

Introduction

Sewage sludge can be an important source of organic matter, micro and macronutrients for the soil. Studies have demonstrated its influence on chemical and physical properties of the soil, as it increases the water holding capacity, the resistance to erosion, improves soil fertility and enhances microbial activity (Boechat, Santos, Accioly, Bomfim, & Santos, 2012). Thus, it has been used for recovery of degraded areas (Bittencourt, Serrat, Aisse, Marin, & Simão, 2012, Sampaio et al., 2012), as a substrate for seedling production (Santos, Kunz, Caldeira, Azevedo, & Rangel, 2014), and in areas cultivated with corn (Barros, Andreoli, Souza Junior, & Costa, 2011), sugar cane (Franco, Marques, & Melo, 2008), eucalyptus (Barreiros, Gonçalves, Sansígolo, & Poggiani, 2007), among other crops.

The total amount of sewage sludge has increased in Brazil, mainly due to population and industrial growth, and to the implementation of new Sewage Treatment Plants (STP). Species and pathogen concentrations in sewage sludge are variable and depend on (United States Environmental Protection Agency [Usepa], 2003, Andreoli, Garbossa, Lupatini, & Pegorini, 2008, Sidhu & Toze, 2009):

- socioeconomic conditions of the population;
- sanitary conditions of the region;
- health of the community served by the STP;
- type of sewage sludge treatment;
- season of the year;
- presence of hospitals and slaughterhouses in the area served by STP.

However, by its origin - from the sewage treatment - the sludge concentrates many elements,

which can pose risks to the environment and public health, such as heavy metals, pharmaceutical contaminants, polyaromatic hydrocarbons and pathogens (Andreoli et al., 2008, Cheng et al., 2012, Jones, Gardner, & Ellor, 2014, Cieslik, Namiesnik, & Konieczka, 2015, Gyawali, Sidhu, Ahmed, Jagals, & Toze, 2015, Rocha, Barés, & Braga, 2016).There is little information on the number and survival of pathogens in biosolids because of difficulties with detection methods, especially for viruses and protozoa (Sidhu & Toze, 2009).

The presence of pathogenic microorganisms in untreated sewage sludge has already been reported by Soccol and Paulino (2000); Paulino, Castro, and Soccol (2001); Graczyk, Lucy, Tamang, and Miraflor (2007); Andreoli et al. (2008); Khouja, Cama, and Xiao (2010); Cheng et al. (2012). In treated sludge, can be cited the studies of Chauret, Springthrope, and Satta (1999) and Soccol and Paulino (2000).

Regarding methods for microbiological analysis of the protozoa *Giardia* and *Cryptosporidium* used in sewage sludge, there is no consensus among authors. In this way, numerous methods are found in the literature, which practically include three main steps: concentration (by sedimentation, centrifugation or filtration); purification (solutions of sodium acetate, acetic acid, zinc sulfate, ethanol or immunomagnetic separation), and detection of (oo)cysts (direct immunofluorescence reaction, FISH - Fluorescence *in situ* hydridization). In this context, it may be mentioned the studies developed by McCuin and Clancy (2005); Graczy et al. (2007, 2008); Ben Ayed, Schijven, Alouini, Jemli, and Sabbahi (2009); Khouja et al. (2010); Konoté, Maiga, Basset, Casellas, and Picot (2013).

According to Sidhu and Toze (2009), given the methodological limitations and the sporadic presence of certain pathogens in biosolids, much of the research focuses on the occurrence of indicator microorganisms. As a result, there is a knowledge gap regarding the potential risk to public health, especially for protozoa and viruses.

In this sense, this study analyzed the presence and determined the viability of cysts of *Giardia* spp. and oocysts of *Cryptosporidium* spp. in sewage sludge from two sewage treatment plants with activated sludge system and checked for differences in concentration and viability of (oo)cysts between activated sludge systems operated conventionally or as extended aeration.

Material and methods

Sampling

Sewage sludge samples were collected from two sewage treatment plants (STP):

- STP - University of São Paulo Campus, in the city of São Carlos, São Paulo State, which has a preliminary treatment, followed by UASB (Upflow Anaerobic Sludge Blanket) and by a pilot plant for activated sludge treatment system. The latter was operated with two solids retention times (SRT), 20 and 7 days, characterizing it as extended aeration and conventional, respectively.
- STP at full scale, located in the municipality of Limeira, São Paulo State, which has preliminary treatment, UASB reactor, conventional activated sludge system and UV radiation disinfection unit.

For this study, 1 liter sludge samples were taken from the return lines of sewage sludge from the activated sludge system of both STPs, using previously washed and disinfected vials, rinsed with (0.1% v v^{-1}) Tween 80 elution solution. Five sludge collections were performed for each SRT at the STP - USP Campus, São Carlos, and four collections at the STP of Limeira, São Paulo State.

Sample processing

First, 50 mL of the sample was centrifuged at 1500 x *g* for 15 min. The supernatant was aspirated and discarded. Into the final pellet, containing less than 5 mL, was added of 10 mL of elution solution - Tween 80 (0.1%). After mixing by vortexing for 30 s, it was again centrifuged (1500 x *g* for 15 min) with supernatant removed and discarded. Subsequently, 10 mL of deionized water were added and other mixing by vortexing. After the third centrifugation (1500 x *g* for 15 min), the supernatant was removed and discarded, remaining a 5 mL final pellet, which was taken to vortex and kept overnight at 7.5 ± 2.5°C.

To quantify the weight of the sample, 50 mL sludge were taken to the drying oven (103-105°C) for analysis of total solids dried: Gravimetric method (American Public Health Association, Water Environment Federation, & American Water Works Association [APHA, WEF, & AWWA], 2005).

Detection and identification of (oo)cysts

From two aliquots of 10 μL pellet left overnight (replication), per sample, the quantification of cysts and oocysts was performed by immunofluorescence assay (IFA) using the Merifluor® kit (Meridien Bioscience Diagnostics, Cincinnatti, Ohio).

Firstly, aliquots were placed on plates and allowed to dry at room temperature (about 30 min).

Then were added 10 to 30 μL methanol and left to dry for 10 min.

After this stage, it was added one drop of the detection reagent and one drop of the counter dye, both present in the Merifluor® kit; and the plates were taken to a moist chamber in the dark at 37°C for 30 min.

For washing, 100 μL deionized water were applied to the plate well, which was tilted to 45º. After, (oo)cysts were analyzed for their viability.

Assessment of (oo)cyst viability

For this, one drop of the dye was applied to each well of the plate, allowing to react for 15 min. After, further washing was performed by applying 100 μL deionized water to the plate well, which was tilted to 45º. It was applied a drop of mounting medium present in the Merifluor® kit, and finally, the cover slip was placed.

The viability of cysts and oocysts was estimated by differential staining with propidium iodide (Sigma-Aldrich®, USA), according to Campbell, Roberston, and Smith (1992). This reagent, responsible for the emission of red fluorescence (λ = 510-550 nm), penetrates only in microorganisms with damaged membrane (dead (oo)cysts).

(Oo)cyst count

The samples were examined under an immunofluorescence microscope (Olympus® BX51) at 400 to 800X magnification.

The concentration of (oo)cysts per gram of dried sludge was calculated according to Equation 1.

$$\text{Number (oo)cysts g}^{-1}\text{ DS} = \frac{\text{\# (oo)cysts counted on the slide} \times \text{FSV}}{\text{SVS} \times \text{WDS} \times 10^{-3}} \quad (1)$$

where:
DS - dried sludge;
FSV - final sediment volume (5 mL);
SVS - sediment volume examined on the slide (10 μL);
WDS - weight of the dried sludge sample (g).

Statistical analysis

Statistical analysis of the data was performed using the software Statistica 7.0 (StatSoft® Inc.). Homoscedasticity was tested by Levene's test and for mean comparison, it was applied ANOVA with Student's t-test. The first hypothesis was that the change in the activated sludge treatment system (conventional to extended aeration) caused differences in the quantification and viability of samples. The second hypothesis is that there are differences between the two studied STPs (São Carlos and Limeira). Differences were considered significant at $p < 0.05$.

Results and discussion

According to Noyola, Padilla-Rivera, Morgan-Sagastume, Guereca, and Hernández-Padilha (2012), activated sludge is the third most widespread technology for the treatment of domestic sewage in Brazil. The aeration system used to maintain the system under aerobiosis causes cysts and oocysts to remain suspended in the liquid mass; subsequently they are transported trapped or not into the biological floc, which is concentrated in the clarifier after the aeration tank.

STP - USP Campus, São Carlos, São Paulo State

At the STP in the USP Campus, in São Carlos, five collections of sludge samples were carried out for each solids retention time (SRT): extended aeration - 20 days - and conventional aeration - 7 days, whose cysts and oocysts counts are listed in Table 1.

Table 1. Concentration and viability of (oo)cysts in sewage sludge from activated sludge treatment system.

SRT	Collection	Cysts of *Giardia* spp. (g^{-1} dried sludge)	Viability (%)	Oocysts of *Cryptosporidium* spp. (g^{-1} dried sludge)	Viability (%)
20 days	I	14008	86.6	ud	-
	II	33806	55.8	3220	100
	III	13475	95.5	1585	50
	IV	21150	92.1	432	100
	V	82143	84.7	1786	100
	Mean±SD	32916±28714	82.9±15.8	17555±1144*	87.5±25*
7 days	I	16824	85.8	ud	-
	II	56417	92.3	ud	-
	III	118474	92.0	4016	100
	IV	17023	96.4	774	100
	V	93805	80.6	ud	-
	Mean±SD	60509±45510	89.4±6.2	2395±2292*	100*

*mean only of samples with oocysts; ud: undetected cysts; SD: standard deviation.

Giardia cysts were found in 100% (10/10) of the samples, while *Cryptosporidium* oocysts were recorded in 60% (6/10) of the samples. Khouja et al. (2010) reported 40% of positive samples for *Giardia* spp. These results were also higher than those reported by Bonatti and Franco (2014). Only for *Cryptosporidium*, the positivity of the samples was lower than that verified by Iacovski, Barardi, and Simões (2004).

Concentrations of cysts were higher than concentrations of oocysts ($p < 0.05$). Comparing the system operated under extended aeration and conventionally, no difference was detected between concentrations of cysts and oocysts ($p > 0.05$).

The significant concentration of cysts and oocysts in the sludge were greater than that found by Graczyk et al. (2008), of approximately 27 *Giardia* cysts and 14 *Cryptosporidium* oocysts per gram of STP sludge and that reported by Bonatti and Franco (2014), of up to 4800 *Giardia* cysts per gram and Gerba, Tamini, Pettigrew, Weisbrod, and Rajagopalan (2011), with concentrations of 10 to 1000 *Giardia* cysts per gram and 100 to 2000 *Cryptosporidium* oocysts per gram.

In agreement with Iacovski et al. (2004), the variation in both the concentration of cysts and oocysts and the percentage of positive samples depends on the characteristics of the population served by the STP, for example, the level of infection of individuals by these protozoa. It is worth mentioning the extrapolation of results, as 10 μL sample are taken for reading a 5 mL of pellet volume, according to Equation 1.

When working with long solids retention time (extended aeration), it is chosen to achieve endogenous respiration in the microbial metabolism, with the advantage that the discarded sludge is stabilized. However, the change, from conventional to extended aeration, caused no significant damage to the wall of (oo)cysts, whereas there was no significant difference in the viability of cysts and oocysts in relation to solids retention times analyzed.

STP–at full scale, municipality of Limeira, São Paulo State

Sludge samples were 100% positive for *Giardia* and 25% for *Cryptosporidium*. Mean concentration and viability of cysts and oocysts found in the sludge of the Limeira STP can be seen in Table 2.

Table 2. Concentration and viability of (oo)cysts in sewage sludge from activated sludge treatment system in STP at full scale.

Collection	Cysts of *Giardia* spp. (g^{-1} dried sludge)	Viability (%)	Oocysts of *Cryptosporidium* spp. (g^{-1} dried sludge)	Viability (%)
I	26158	88.1	ud	-
II	63895	91.3	3600	100
III	17330	93.6	377	50
IV	23881	94.8	ud	-
Mean±SD	32816±21055	92±2.9	1989±2279*	75±25*

*mean only of samples with oocysts; ud: undetected cysts; SD: standard deviation.

Mean values of cysts and oocysts were close to those found at the STP - USP São Carlos. In these cases, there were no statistical differences between the mean values and percentages of viability of the two STP - at full scale and at pilot scale.

Graczyk et al. (2007) found concentrations of up to 114 *Giardia* cysts and 110 *Cryptosporidium* oocysts in sludge cake from activated sludge system. The same authors observed that most (oo)cysts counted were viable (> 99%). Graczyk et al. (2008) registered oocysts 14 oocysts and 26 cysts g^{-1} of *Cryptosporidium* and *Giardia*, respectively, much lower than the concentrations obtained in this study.

Additionally, the comparison of our results with other studies is difficult due to different methodologies, inconsistent sampling and different types of sludge. The pathogenic protozoa *Giardia* and *Cryptosporidium* are not product from sludge characterization, according to Conama Resolution, no. 375 (Brasil, 2006), unless otherwise requested by the competent environmental agency.

For a better estimate of the risk associated with the use of sludge, one of the first steps is the hazard characterization, which characterizes and quantifies chemical contaminants and pathogenic microorganisms (Cheng et al., 2012, Jones et al., 2014).

The results raise concerns due to contamination of sludge intended for application to the soil, requiring treatment. This concern should be extended to other types of sludge from the treatment of other wastewaters that may have a high concentration of pathogens (pig and cattle raising; slaughterhouses etc.).

Regarding that agriculture is one of the main destinations of sewage sludge, it should be highlighted the direct and indirect risks, such as contamination of soil, water, food, and toxic effects on crops. Besides that, attention should be given to sludge management to preserve the health of workers and other exposed populations.

It is worth mentioning that the Conama Resolution, n. 375 (Brasil, 2006) does not include (oo)cysts of *Giardia* and *Cryptosporidium* as microbiological standard for biosolids Class A and B for use in agriculture. Bastos, Bevilacqua, and Mara (2013) pointed out that, for a review and updating of this resolution and also with a view to the use of quantitative assessment of microbiological risk of biosolids, in which the concentration data like those found in this work become essential.

Conclusion

The change in the activated sludge operation system, from extended aeration to conventional, at the STP - USP São Carlos, caused no difference in the concentration and viability of (oo)cysts of protozoa analyzed in the sludge.

The concentrations of *Giardia* cysts and *Cryptosporidium* oocysts observed in the sludge in both plants highlight the potential risks to human health in the event of use of untreated sewage sludge, because much is still viable, which

emphasizes the need for treating sewage sludge before use.

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