Real scale environmental monitoring of zoonotic protozoa and helminth eggs in biosolid samples in Brazil

Taís Rondello Bonatti · Regina Maura Bueno Franco

Received: 21 February 2014 / Accepted: 25 August 2014 / Published online: 5 September 2014
© Indian Society for Parasitology 2014

Abstract Biosolid is the product of the activated sludge treatment system and its final disposition is subject of ongoing discussion as this residue can therefore harbor a great number and variety of pathogens. This study was aimed to (1) monitor the presence of *Giardia* and *Cryptosporidium* in biosolid samples from a treatment plant in Campinas, SP, Brazil, (2) observe *Giardia* cyst wall morphological integrity in treated samples using scanning electron microscopy (SEM) and (3) verify the presence and viability of helminth eggs. Cysts were present in 33.3 % of the samples, whereas oocysts were detected in 8.3 %. Viable *Ascaris* sp. *Toxocara* sp. and similar to *Trichuris* sp. eggs were found through the use of Mexican Official Norm. Results demonstrate the difficulties inherent in working with biosolid as factors such as temperature, ionic strength and pH influenced the recovery of cysts and oocysts. Pores and ruptures were not observed in cyst wall visualized by SEM following 45 days of exposure to sunlight, only minimal morphological changes. These observations emphasize both the importance of adequate treatment of sewage sludge and the need to develop appropriate techniques for the detection of *Giardia* and *Cryptosporidium* in this type of sample. This is the first time that a study was done in a real scale for biosolid samples in determining the presence of pathogenic protozoa as *Giardia* and *Cryptosporidium* in Brazil, and also

observed minimal cyst wall damage after sunlight treatment.

Keywords *Giardia* · *Cryptosporidium* · Helminth eggs · Biosolid · Monitoring

Introduction

Biosolid produced by the activated sludge treatment system is a material rich in nutrients and organic matter that may contain pathogens such as viruses, bacteria and parasites that are capable of surviving under adverse environmental conditions (temperatures variations, pH changes and humidity, among others), even after treatment and processing (USEPA 1999).

With the population increase and urbanization, biosolid production also has increased and the application of this residue to soil for fertilization purpose represents the best option for disposal as this is rich in nutrients (Guadagnini et al. 2013; Hachich et al. 2013; Lima et al. 2012; Lu et al. 2012; Silva et al. 2012). However, inherent in its use in agriculture are concerns about its effects on public health due to uncertainties surrounding the presence of enteric parasites, which can be sources of contamination in both food and surface water and groundwater; the low infective dose of certain pathogens, especially *Cryptosporidium* and *Giardia*; the possible growth of pathogenic bacteria, such as coliforms, which are used as indicators of safety but do not predict the presence of other pathogens (Abreu-Acosta and Vera 2011); and the potential for diseases caused by unknown agents (Santamaria and Toranzos 2003, Sidhu and Toze 2009).

Moreover, studies suggest that factors such as temperature, humidity and pH may not be sufficient for the

T. R. Bonatti (✉) · R. M. B. Franco
Animal Biology Department, Biology Institute, State University of Campinas, PO Box 6109, Campinas, SP CEP: 13083-970, Brazil
e-mail: taisbonatti@yahoo.com.br

R. M. B. Franco
e-mail: mfranco@unicamp.br

inactivation of these forms, depending on the level of contamination (Jiménez et al. 2002, King and Monis 2007) and given the ability of these organisms to survive adverse environmental conditions, the absence of a specific and standardized methodology for determining the presence of certain pathogens further underscores the need for additional study of biosolid. These aspects show a great potential impact of this usage in the environment (Silva et al. 2012).

Investigations into the frequency with which *Giardia* cysts and *Cryptosporidium* oocysts can be observed in biosolid samples are therefore relevant from a public health perspective. Additionally, there is a paucity of data in Brazil regarding the presence of pathogenic protozoa in this type of waste, and there is no standardized methodology for their detection.

Helminth infections are a major public health concern in developing countries, especially in tropical and subtropical regions of Africa, Asia, Central and South America (WHO, WHO 2006). Additionally, helminth eggs are considered one of the main biological health risks when applying biosolids on agricultural soils and these parasites were included in the present research as Brazil has about 41.7 million people harboring *Ascaris lumbricoides* (Guadagnini et al. 2013; Navarro et al. 2009; Pinheiro et al. 2011).

In this study we report that *Giardia* spp. cysts found in biosolid samples exhibit minimal damage after 45 days sunlight exposure treatment as measured by Scanning Electronic Microscopy (SEM) as well as viable *Ascaris* spp. eggs. These findings are particularly relevant due to the hot and humid climate in Southeast Brazil that contributes for the survival and spread of these parasites. Also, such factors can contribute for soil and water contamination where the biosolid is applied to.

Materials and methods

Sampling site

Biosolid samples were collected at Opersan Company in Jundiaí, São Paulo (23°8'55"S; 46°53'29"W), which is responsible for the treatment of biosolid generated via the activated sludge process by the Jundiaí Sanitation Company and the Samambaia and Santa Rosa wastewater treatment plants (WWTP) in Campinas. This plant at Jundiaí treats 200 tons of biosolid per day. In this study, samples were collected from the Samambaia WWTP, which has an average flow of 98 L/s and is responsible for the treatment and removal of the sewage from approximately 40,000 people, with a production of 5 tons of biosolid per day (SANASA 2011). Each pile had 4 m base and 1.5 m high. Biosolid sample collection was conducted to

obtain a composite sample that encompassed various portions of the compost pile. For this, a portion from the upper, middle and from the bottom as well as from the two ends were collected until reaching to 500 g in a depth of 50 cm.

Sampling

The biosolid samples were collected at the time of the disposal of sludge in the compost pile (t_0) and following 45 days (t_{45}) and 90 days (t_{90}) of sun exposure between January 2005 and January 2006, with a total of 12 samples taken from four piles, three samples from each (Fig. 1; Table 1). So, the samples were collected every 45 days during a 1 year period. For each sample, a test was performed to evaluate the sensitivity of the methodology used (a positive control) by processing a sample that had been artificially contaminated with known amounts of cysts and oocysts (Branco et al. 2012). This sensitivity control was processed simultaneously with the raw sample.

Samples were collected in plastic bottles that had been previously decontaminated and washed with an elution solution containing 0.01 % Tween 80–50 mM TRIS (Tris(hydroxymethyl)aminomethane) in distilled water. The samples were then transported to the laboratory in a closed container maintained at 4 °C.

Each sample was divided into two aliquots: one was subjected to the evaluation of cysts and oocysts to observe naturally occurring parasites, and the other was artificially contaminated (positive control). The samples were divided and then processed simultaneously according to the following procedure:

Homogenization of the sample of biosolid with elution solution

This procedure was performed as described by Redlinger et al. (2002) with modification regarding that TRIS was added to the elution solution. This salt improves the dispersion of particles within the sample (Lindahl and Bakken 1995, Udeh et al. 2003).

An amount of 11 g sludge was placed in a previously decontaminated beaker and was added 99 mL of 0.01 % Tween 80–50 mM Tris in distilled water. Then, it was shaken vigorously by hand, using a glass rod to homogenize the sample (2 min). An aliquot of 5 µL of the resulting solution was analyzed by direct immunofluorescence assay (IFA) after homogenization in vortex (2 min).

Quantification of cysts and oocysts in the samples

The kit used was Merifluor (Meridian Bioscience Diagnostics, Cincinnati, Ohio, USA). The immunofluorescence

Fig. 1 Biosolid compost piles. **a** Compost pile at the moment that sewage sludge is disposed, with 0 days of sun exposure. **b** Compost pile after 90 days of sun exposure

Table 1 Schedule of biosolid sample collections for 90 days during 1 year study

Compost pile	Sampling schedule			
	CP1	CP2	CP3	CP4
Months	Jan/05 to Apr/05	Apr/05 to Jul/05	Jul/05 to Oct/05	Oct/05 to Jan/06
Samples	RS + PC $t_0^a, t_{45}^b, t_{90}^c$	RS + PC t_0, t_{45}, t_{90}	RS + PC t_0, t_{45}, t_{90}	RS + PC t_0, t_{45}, t_{90}

CP compost pile, RS raw sample, PC positive control

^a t_0 Sample taken at the time of disposal in the compost pile

^b t_{45} Sample taken after 45 days of exposure to the sun

^c t_{90} Sample taken after 90 days of exposure to the sun

microscope used has a 450–490 nm excitation filter and a barrier filter of 520 nm and positive criteria was observed according (USEPA 2005).

To calculate the number of cysts and oocysts per gram we considered the number of (oo)cysts observed in the immunofluorescence slide well, the sample mass used to perform the procedure and the sediment volume placed in the slide well (μL) according to the following equation:

$$x = \frac{n}{K \times A \times 10^{-3}} \quad (1)$$

where x is the concentration of cysts or oocysts per g; n is the number of cysts or oocysts viewed; K is 5 (the volume of sediment examined = 5 μL); A is the mass of the sample used (11 g) and 10^{-3} is the correction factor for 100 mL (the final volume obtained).

Artificially contaminated samples with cysts and oocysts (positive control)

All biosolid aliquots were artificially contaminated using a purified suspension containing a known concentration of cysts and oocysts and then subjected to the same procedures outlined above (Parasitological Analysis), according

to the procedure as described by Branco et al. (2012). The average inoculum concentration used when artificially contaminating the samples followed a predetermined order of magnitude: 10^4 for cysts and 10^3 for oocysts.

The recovery efficiency (Y) for cysts and oocysts was calculated as follows:

$$y = \frac{r \times 100}{i} \quad (2)$$

where y is the recovery efficiency (%); r is the number of cysts or oocysts recovered (deducted from the number of cysts and oocysts naturally found in raw samples); and i is the total number of cysts or oocysts expected in 5 μL .

Giardia cysts purification

Positive faecal samples for *Giardia* cysts were purified according to the protocol developed by Smith and Smith (1989) with the following modifications regarding the volumes of the sucrose gradient: 1.02, 1.06 and 1.18 g/mL. This was only used as a positive control since it was recently eliminated and purified from faeces, in order to compare the morphological damage on cysts found under natural conditions in biosolid samples by SEM.

Scanning electron microscopy (SEM)

This procedure was performed according to the methodology of Widmer et al. (2002). Coverslips were prepared with poly-L-lysine for 1 h before the procedure.

A 10 µL aliquot pre-fixed in 2.5 % glutaraldehyde was placed onto a coverslip and washed in phosphate buffer (sterile PBS) for three times for 10 min. Then, it was performed a post-fixation in 1 % osmium tetroxide (OsO₄) for 1 hour (room temperature, protected from light) and subsequently washed in phosphate buffer solution for 10 min. The next steps consisted in dehydration in ethanol series of increasing concentrations: 30, 50, 70, 90 and 100 % (three times) for 10 min.

After critical point drying, samples were coated with gold and examined in order to observe damage in the cysts surfaces, including pores, folds, grooves, deformations, membrane disruption that could result in a loss of infectivity.

Determination of pH

To measure the sample pH, 40 mL of Milli Q water was added to a 10 g sample of biosolid for all samples, except for the first (t₀) and second sample (t₄₅) from the compost pile 1. As the results were negative for these samples, we opted to verify pH. The suspension was homogenized with the aid of a magnet on a shaker for 15 min and then the pH was measured using a calibrated the pH meter (Model Q-400A, Quimis) (Cantusio Neto et al. 2010).

Detection of *Ascaris* eggs and other helminths

This procedure was performed as described by (Mexican Official Norm 1996) with modification on the initial mass: it was used 30 g rather than 10 g of sample. This method has an initial concentration step by natural sedimentation combined with a biphasic solution (alcohol—acid solution; 0.1 N H₂SO₄ in 33–35 % C₂H₅OH), followed by extraction with a flotation solution (zinc sulphate).

Viability of helminth eggs

In order to test the viability of helminth eggs, we performed the Trypan Blue exclusion test, as described by (Victorica and Galvan 2003). An aliquot of 6 µL of the final pellet was placed on a slide along with 6 µL of Trypan Blue dye (0.1 %). Visualization was made between 3 and 5 min after making the preparation according to Altman et al. (1993). Viability determination is not indicated in the description of the NOM method (Victorica and Galvan 2003).

Artificially contaminated samples with *Ascaris suum* eggs (positive control)

For obtaining the purified suspension of helminth eggs of *Ascaris suum*, females were dissected as per the method described/used by Jensen et al. (2009). The females were obtained from pig farms of Pomerode city, Santa Catarina, Brazil and *Ascaris suum* was used as a surrogate of *Ascaris lumbricoides*. Eggs were recovered from the female uteri and, after 48 h sedimentation, they were recovered from the recipient bottom and enumerated for the inoculum estimative.

Three raw sewage samples were artificially contaminated using a purified suspension containing a known concentration of *Ascaris suum*, with the characteristic external coat, embryonated and with larvae, and then subjected to (Mexican Official Norm 1996).

The recovery efficiency (Z) for eggs was calculated as follows:

$$z = \frac{r \times 100}{i} \quad (3)$$

where Z is the recovery efficiency (%); r is the number of eggs recovered (deducted from the number of eggs naturally found in raw samples); and i is the total number of cysts or oocysts expected in 50 µL.

Statistical analysis

The statistical analyses were performed using the Student t test to compare the samples positivity and sampling time (P value < 0.05). The ability to detect the presence of *Giardia* cysts and *Cryptosporidium* oocysts and the efficiency with which they could recover (oo)cysts were evaluated.

Results

Occurrence of *Giardia* cysts and *Cryptosporidium* oocysts

Giardia cysts were identified for a total of four positive samples (33.3 %) (Table 2). The average concentration of cysts per gram of biosolid detected in the four positive samples was 600 cysts/g (ranging from 0 to 4,800 cysts/g). For *Cryptosporidium*, only one sample from the compost pile 2 (t₄₅) was positive (8.3 %), with a concentration of 600 oocysts/g.

When an analysis was performed to compare the different sampling times over a period of 1 year it was not identified a pattern of increased or decreased concentrations

Table 2 Concentration of *Giardia* cysts and *Cryptosporidium* oocysts per gram of biosolid in raw samples and recovery efficiencies of control experiments (%)

Procedure		CP 1			CP 2			CP 3			CP 4			Mean	SD
		t ₀ ^a	t ₄₅ ^b	t ₉₀ ^c	t ₀	t ₄₅	t ₉₀	t ₀	t ₄₅	t ₉₀	t ₀	t ₄₅	t ₉₀		
Raw sample	<i>Giardia</i>	0	0	0	1,200	0	0	600	4,800	0	0	0	600	600	1319.1
	<i>Cryptosporidium</i>	0	0	0	0	600	0	0	0	0	0	0	0	50	165.8
Control	<i>Giardia</i>	0	0	0	0	0	0	19.4	0	70.8	7.8	0	0	8.2	19.7
	<i>Cryptosporidium</i>	0	0	0	0	1.4	0	5.6	9.2	0	3.9	0	0	1.7	2.9

CP compost pile, SD standard deviation

^a t₀ Sample taken at the time of disposal in the compost pile

^b t₄₅ Sample taken after 45 days of exposure to the sun

^c t₉₀ Sample taken after 90 days of exposure to the sun

Fig. 2 *Giardia* cysts visualized with Scanning Electronic Microscopy. **a** *Giardia* cyst from control sample (hospital sewage, artificially contaminated) ($\times 3,000$). **b** *Giardia* cyst from compost pile 4, t₄₅. ($\times 3,000$)

of *Giardia* cysts per gram of biosolid for the four piles analyzed (Table 2).

Artificially contaminated samples for cysts and oocysts (positive control)

For *Giardia* cysts, the recovery efficiency ranged from 7.8 to 70.8 % (mean 6.8 %). For *Cryptosporidium* oocysts, the recovery efficiency ranged from 1.4 to 9.2 % (mean 2.3 %), (Table 2).

Evaluation of cysts morphological damage by direct visualization using SEM

In this work, SEM performed with samples from compost pile 4 compared with control samples (effluent hospital artificially contaminated with a suspension of purified *Giardia* cysts) suggests that, after 45 days of exposure to the sun, the cysts still maintain its integrity.

Cyst wall showed minimal morphological changes as ruptures, vacuolization and disruption of the integrity were not observed (Fig. 2a,b) even after 45 days of sun light exposure.

Determination of pH

The pH values of samples are shown in Table 3. The variation was from 5.25 to 8.44 at different sampling times.

Helminth eggs detection

Ascaris sp. eggs were detected in two samples ($n = 2/12$, 16.6 %), with a concentration from 0 to 11 eggs/g; *Toxocara* sp. eggs, in five samples ($n = 5/12$, 41.6 %) with a range from 0 to 22eggs/g and *Trichuris* eggs, in one sample ($n = 1/12$, 8.3 %), ranging from 0 to 11 eggs/g (Table 4).

Ascaris spp. eggs were viable by Trypan Blue exclusion test (Fig. 3a,b). For *Toxocara* spp. one sample showed no

Table 3 pH values and mean measured for samples biosolid for the four compost piles

	CP 1	CP 2	CP 3	CP 4
t ₀ ^a	NM	7.92	8.18	8.14
t ₄₅ ^b	NM	7.56	8.44	7.84
t ₉₀ ^c	6.7	5.25	6.73	5.57
Mean	6.7	6.91	7.78	7.18
SD	3.9	1.4	0.9	1.4

NM not measured, CP compost pile, SD standard deviation

^a t₀ Sample taken at the time of disposal in the compost pile

^b t₄₅ Sample taken after 45 days of exposure to the sun

^c t₉₀ Sample taken after 90 days of exposure to the sun

Table 4 Helminth eggs per gram concentration on biosolid samples

Compost pile	Sample	Eggs/g		
		<i>Ascaris</i>	<i>Toxocara</i>	<i>Trichuris</i>
1	t ₀	0	0	0
	t ₄₅	0	0	0
	t ₉₀	0	22	0
2	t ₀	0	0	0
	t ₄₅	11	11	0
	t ₉₀	0	11	0
3	t ₀	0	0	0
	t ₄₅	0	0	0
	t ₉₀	0	0	0
4	t ₀	0	0	11
	t ₄₅	0	22	0
	t ₉₀	11	11	0

dye inclusion (Fig. 4); for *Trichuris* spp., eggs were not viable (Fig. 5).

Artificially contaminated samples for helminth eggs (positive control)

The recovery efficiency ranged from 49.1 to 75.3 % (mean 60.87 %).

Discussion

The concentration of *Giardia* cysts found in this study (0 to 4800 cysts/g), corroborates what was reported by Hu et al. (1996) (0 to 7000 cysts/g) in biosolid samples. Other researchers have likewise observed high concentrations of cysts per gram of biosolid and final effluent. Thiriart et al. (1997) found concentrations varying from 0 to 8700 cysts/g of wet biosolid; Lemarchand and Lebaron (2003) reported concentration ranging from 0 to 209.2 oocysts/L in

samples of activated sludge; Jiménez et al. (2002) observed concentrations of 130 and maximum of 4,200 cysts/g in samples of biosolid. Bonatti et al. (2007) found *Giardia* concentrations ranging from 0 to 4.8×10^5 cysts/L in samples of activated sludge from the Samambaia WWTP (Campinas, São Paulo, Brazil).

For oocysts, the number of positive samples identified in this study (8.3 %) was lower than has been reported in previous studies in other countries (Jiménez et al. 2002, Lemarchand and Lebaron 2003, Thiriart et al. 1997). This may be explained by several factors, including the prevalence of cryptosporidiosis in those communities and the individual characteristics, such as pH, of the sludge matrix studied.

According to Guzmán et al. (2007), data reported that *Cryptosporidium* is present in non-negligible amounts and should be considered as a potential health risk. These authors also comment that it is impossible to precise the removal of these pathogens in such highly inactivating processes like thermal treatments, liming or composting.

Cryptosporidium oocyst has a low hydrophobicity and this can be increased by suspensions with high conductivity, which can influence adhesion to hydrophobic surfaces. Understanding the phenomenon of adsorption involves the evaluation of both the hydrophobicity and electrostatic surface characteristics of the parasite. Hydrophobicity is a parameter that, when correlated with chemical characteristics and structure of the parasite, has a significant influence on the mechanisms of adhesion of the oocyst and their association with particulate matter (Drozd and Schwartzbrod 1996).

Daí et al. (2004) reported the adhesion of cysts and oocysts to four materials of different surface charges and hydrophobicities and also that, in contrast to the cysts, adhesion is governed primarily by surface charge for oocysts. Because the oocysts are negatively charged, repulsive electrostatic forces that increase the load of inorganic particles become more negative. This effect prevents the adhesion of oocysts to the sediment and decreases their removal by sedimentation (Searcy et al. 2005). With regard to cysts, data suggest that hydrophobicity is more important than surface membrane charge on solid surfaces.

According to Searcy et al. (2005), the biological oocysts are spherical colloids with a surface composed of a complex array of glycoproteins. These structures have a negative surface charge under typical environmental conditions, likely due to the presence of carboxylate groups, carboxylic acid and phosphate on their surfaces. Both the electrostatic and steric forces can act in association with suspended oocysts.

Cook et al. (2006) stated that a standard method for the detection of protozoa should be relatively simple, robust, reproducible, fast, reliable and realistic. These authors

Fig. 3 *Ascaris* sp. eggs. **a** Viable *Ascaris* sp. egg found in compost pile 4, sample t_{90} (did not incorporate Trypan Blue dye). **b** Non-viable *Ascaris* sp. egg found in compost pile 4, sample t_{90} (incorporated Trypan Blue dye) ($\times 250$)

Fig. 4 *Toxocara* sp. eggs **a** and **b** Non-viable *Toxocara* sp. Egg found in compost pile 1, sample t_{90} (incorporated Trypan Blue dye) ($\times 480$)

Fig. 5 Egg similar to *Trichuris* sp. viable. Found on compost pile 4, sample t_0 (did not incorporate Trypan Blue dye) ($\times 250$)

noted that during the standardization and optimization of a technique, it is important to identify interfering factors that may reduce recovery or increase variability. For this

reason, in the case of this study, TRIS salt was added to the elution solution, one of the best agents for dispersion (Lindahl and Bakken 1995).

With regard to the morphological integrity of the cysts wall, we observed that it had a wilting aspect but it was not observed pores or a rupture of this structure even after 45 days of sun exposure. Only minimal morphological changes, including a limited number of grooves and deformations in the cyst wall were seen, with no noticeable damage or shrinkage signs (Fig. 2). Widmer et al. (2002) reported that structural damage including wall disruption visualized by SEM in cysts exposed to ozone treatment may be related to the loss of infectivity. In that study, damage was observed in preparations that may impair the integrity of the cyst wall (not observed in the present study). The lack of damage to *Giardia* (found under natural conditions in the sample t_{45}) observed by SEM suggests that the parasite is highly resistant to adverse environmental conditions. However, proof of infectivity can be obtained only by animal infectivity tests but, due to the

complexity of the sample, the sanitization of the inoculum would be an extremely difficult step. SEM is a faster technique, and these considerations led to the selection of SEM as a method to evaluate the cyst integrity. The presence of an intact and functional cyst wall, maintaining the intracellular environment, is one of the main pre-requisite for viability (Hammes et al. 2011).

It was observed a wide variation in the pH (from 5.25 to 8.44), which may also explain the variability of the results of the control experiments for protozoa resistance forms. Based on multivariate analysis, Bonadonna et al. (2002) found a significant correlation between the number of *Cryptosporidium* oocysts and pH. The concentration of oocysts in the effluent was higher at lower pH values. In the present study, oocysts were observed in a single sample with a pH of 5.25 (the third sample of the second compost pile), the lower pH measured among the samples.

The difference in geographic locations should also be taken into consideration because the prevalence of parasitic diseases differs in various communities with respect to both symptomatic and asymptomatic individuals (Robertson et al. 2006). According to Franco (1996), in a study of children aged 2 to 42 months attending daycare in the city of Campinas, SP, the prevalence of *Giardia duodenalis* was 10.09 %. Rimhanen-Finne et al. (2004) stressed that knowledge about the presence of *Giardia* and *Cryptosporidium* in samples of biosolid is limited. They also emphasized the importance of continuously revolving the piles and of monitoring temperature to achieve the ideal temperature for the inactivation of pathogens.

The dark color that is characteristic of the biosolid even before its application to soil may influence the detection of oocysts. Johnson et al. (1997) found that oocysts survive longer in the dark than in sunlight, and surpass the survival of other pathogens such as poliovirus, *Giardia* and *Salmonella*. In sewage treatment plants in the region of Campinas, the sludge is typically stored for 15 days before being treated to produce biosolid for agricultural use. Therefore, there is the potential risk to survival at least a portion of the pathogens.

The Mexican Official Norm, in force since January 1997, sets helminth eggs limit that may be present in waste water and sewage. However, it does not intend to evaluate the viability of such eggs, which is fundamental to the study of the epidemiology of parasitic infections (Victorica and Galvan 2003). (Victorica and Galvan 2003) evaluated the efficiency of recovery of this technique and obtained 90 % for helminth eggs in samples of influent, effluent and sludge. The USEPA determined that the detection limit is 1 viable helminth egg per 4 g of sewage sludge (dry weight) (Capizzi-Banas et al. 2004). In this study, we choose not to use the method described by USEPA (USEPA 1999), for checking the viability recommended as this includes the

incubation of the biosolid during three or 4 weeks and this is biased by fungi proliferation, while the Mexican Official Norm did not contemplate such a step, which is of fundamental importance in the epidemiology of parasitic infections (Victorica and Galvan 2003). Therefore, we opted for an alternative verification through the use of Trypan Blue exclusion test because it is an ease and fast procedure.

Ascaris eggs were observed by Trypan Blue exclusion test, which shows the potential risk of contamination from sewage sludge even after 45 days exposure to sunlight. Jiménez et al. (2002) assessed the viability of *Ascaris* eggs in sewage sludge samples in Mexico by employing purification technique with steps of washing, filtration, flotation in saturated and centrifugal concentration and then incubated for 20 days at 26 °C sulfuric acid (0.05 mol/L). According to the authors, *Ascaris* eggs were found more frequently when compared to other helminth whereas 90 % of them were viable. It is unlikely that in the case of this study, the viability has been underestimated since it was made ready ova visualization after staining with Trypan Blue (3 to 5 min), according to Altman et al. (1993).

In Brazil, sewage networks and WWTP construction have expanded in recent years according to Brazilian Institute of Geography, to overcome a historical deficiency in this sector but, despite this growing, among the most important environmental problems are the generation and disposal of sewage sludge, main residue of the treatment (Heller 2007; Silva et al. 2012).

According Lapen et al. (2008), conventional agricultural practices such as fertilizing crops with organic fertilizers like biosolids can be a source of fecal pollution to adjacent surface water bodies and the implications of drainage management upon downstream pathogen levels and associated human infection risks should be elucidated.

Additionally, most research worldwide into biosolid has been conducted through laboratory studies, in which environmental conditions are controlled (O'Connor et al. 2005). Such studies are effective in investigating the role of individual factors however, work by O'Connor et al. (2005) highlights the limitations of extrapolating these studies to conditions outside the context of experimental environment. The present study was conducted on a real scale, in the absence of controlled environmental conditions, with the objective of verifying the presence of cysts and oocysts under natural conditions, monitor cysts wall damage and, also, verify helminth eggs viability.

The occurrence of diseases caused by viruses, protozoa and helminth pathogenic shows that the traditional indicator bacteria are unsuitable as the only indicators for safe disposal of sludge due to strong resistance to various traditional processes of removal as well as protozoa and helminths destruction (Jiménez et al. 2002; Guadagnini et al. 2013). The use of helminth eggs as an indicator to

monitor the presence of pathogens in biosolid is based on the premise that present high rate of survival (years) compared to bacteria (months) and protozoa (months) and resistance to chlorination processes and high temperature (Jenkins et al. 1998, Shamma and Al-Adawi 2002).

Also, in Brazil, as well as in the developing world, there are few data available on the role of biosolid as a reservoir for parasitic protozoa, despite growing interest in the use of this residue in agricultural soil (Anceno et al. 2007). Also, the application in agricultural soils can be geochemically sustainable method to eliminate the disposal problem in urban areas (Silva et al. 2012). In 2006 and again in 2011, CONAMA (National Environmental Council) issued a legal norm (430/2011) that set limits on the presence of helminth eggs, *Salmonella*, fecal coliform and viruses in biosolid used for agricultural purposes. In Sao Paulo, CETESB (Society of Environmental Sanitation Technology) issued an ordinance (P.4.230) establishing conditions for the quality control and quantity of biosolid that can be used for agriculture, but these standards do not cover *Giardia* and *Cryptosporidium* (CETESB 1999).

We can infer from the results and other published studies that, even though there is an incentive of the biosolid land application, there are still restrictions. Data from SEM suggest that there is minimum damage to cysts wall and this form can remain viable even after sunlight treatment. Also, the use of a simple technique, as employed in the present study, can help developing countries to assess biosolid as Immunomagnetic Separation has a prohibitively cost in those countries (Anceno et al. 2007).

The authors conclude that *Giardia* cysts and *Ascaris* eggs represent a potential threat for Public Health considering the biosolid amount produced per year (around 150–220 tons), the increase on the agricultural soil application in Brazil and the negligence in Brazilian legal norms presented until this moment.

Acknowledgments We would like to thank Sanasa (Sanitation and Water Supplying Society, Campinas) and Opsan Company at Jundiaí that allowed the sampling and provided the samples. Also, we would like to thanks CAPES (Coordination of Improvement of Higher Education Personnel) for the project funding.

References

- Abreu-Acosta N, Vera L (2011) Occurrence and removal of parasites, enteric bacteria and faecal contamination indicators in wastewater natural reclamation systems in Tenerife-Canary Islands, Spain. *Ecol Eng* 37:496–505
- Altman SA, Randers L, Rao G (1993) Comparison of Trypan Blue dye exclusion and fluorometric assays for mammalian cell viability determinations. *Biotechnol Prog* 9:671–674
- Anceno A, Katayama H, Houtp E, Chavalitshewinkoon-Petmitr P, Chuluun B, Shipin OV (2007) IMS-free DNA extraction for the PCR-based quantification of *Cryptosporidium parvum* and *Giardia lamblia* in surface and waste water. *Int J Environ Heal R* 17:297–310
- Bonadonna L, Briancesco R, Ottaviani O, Veschetti E (2002) Occurrence of *Cryptosporidium* oocysts in sewage effluents and correlation with microbial, chemical and physical water variables. *Environ Monit Assess* 75:241–252
- Bonatti TR, Franco RMB, Cantusio NR (2007) Comparison of two methodologies for detection of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in activated sludge samples from a sewage treatment plant in the city of Campinas, São Paulo State Brazil. *J Water Health* 5:609–614
- Branco N, Leal DAG, Franco RMB (2012) A parasitological survey of natural water springs and inhabitants of a tourist city in Southeastern Brazil. *Vector Borne Zoonotic Dis* 12:410–417
- Cantusio Neto R, Santos LU, Sato MIZ, Franco RMB (2010) *Cryptosporidium* spp. and *Giardia* spp. in surface water supply of Campinas Southeast Brazil. *Water Sci Technol* 62:217–222
- Capizzi-Banas S, Deloge M, Remy M, Schwartzbrod J (2004) Liming as an advanced treatment for sludge sanitisation: helminth eggs elimination—*Ascaris* eggs as model. *Water Res* 38:3251–3258
- CETESB—Company of Technology of Environmental Sanitation (1999) Application of sewage sludge from biological handling systems in agricultural areas: criteria for project and operation: Technical Manual, Norm P 4230, Sao Paulo
- CONAMA National Environmental Council (2011) Resolution no. 430. Brasília, Brazil
- Cook N, Paton CA, Wilkinson N, Nichols RAB, Baker K, Smith HV (2006) Towards standard methods for the detection of *Cryptosporidium parvum* oocysts on lettuce and raspberries. Part 1: development and optimization of methods. *Int J Food Microbiol* 109:222–228
- Daí X, Boll J, Hayes ME, Aston DE (2004) Adhesion of *Cryptosporidium parvum* and *Giardia lamblia* to solid surfaces: the role of surface charge and hydrophobicity. *Colloid Surf B* 34:259–263
- Drozd C, Schwartzbrod J (1996) Hydrophobic and electrostatic cell surface properties of *Cryptosporidium parvum*. *Appl Environ Microb* 62:1227–1232
- Franco RMB (1996) Giardiasis and cryptosporidiosis in daycares at Campinas, SP. *Rev Soc Bras Med Tro* 29:585–591
- Guadagnini R, Santos L, Franco R, Guimarães J (2013) Inactivation of bacteria and helminth in wastewater treatment plant effluent using oxidation processes. *Water Sci Technol* 68(8):1825–1829
- Guzmán C, Jofre J, Montemayor M, Lucena F (2007) Occurrence and levels of indicators and selected pathogens in different sludges and biosolids. *J Appl Microbiol* 103:2420–2429
- Hachich EM, Galvani AT, Padula JA, Stoppe NC, Garcia SC, Bonanno VMS, Barbosa MRF, Sato MIZ (2013) Pathogenic parasites and enteroviruses in wastewater: support for a regulation on water reuse. *Water Sci Technol* 67:1512–1518
- Hammes F, Berney M, Egli T (2011) Cultivation-independent assessment of bacterial viability. *Adv Biochem Engin/Biotechnol* 124:123–150
- Heller L (2007) Basic Sanitation in Brazil: lessons from the past, opportunities from the present, challenges for the Future. *J Comp Soc Welf* 2:141–153
- Hu CJ, Gibbs RA, Mort NR, Hofstede HT, Ho GE, Unkovich I (1996) *Giardia* and its implications for sludge disposal. *Water Sci Technol* 34:179–186
- Jenkins MB, Bowman DD, Ghiorse WC (1998) Inactivation of *Cryptosporidium parvum* oocysts by ammonia. *Appl Environ Microb* 64:784–788
- Jensen PKM, Phuc PD, Konradsen F, Klank LT, Dalsgaard A (2009) Survival of *Ascaris* eggs and hygienic quality of human excreta in Vietnamese composting latrines. *Environ Health* 8:57–65

- Jiménez B, Maya C, Sánchez E, Romero A, Lira L, Barrios JA (2002) Comparison of the quantity and quality of the microbiological content of sludge in countries with low and high content of pathogens. *Water Sci Technol* 46:17–24
- Johnson DC, Enriquez CE, Pepper IL, Davis TL, Gerba PC, Rose JB (1997) Survival of *Giardia*, *Cryptosporidium*, poliovirus and *Salmonella* in marine waters. *Water Sci Technol* 35:261–268
- King BJ, Monis PT (2007) Critical processes affecting *Cryptosporidium* oocysts survival in environment. *Parasitology* 134:309–323
- Lapen DR, Topp E, Edwards M, Sabourin L, Curnoe W, Gottschall N, Bolton P, Raman S, Ball-Coelho B, Payne M, Kleywegt S, McLaughlin N (2008) Effect of liquid municipal biosolid application method on tile and ground water quality. *J Environ Qual* 37:925–936
- Lemarchand K, Lebaron P (2003) Occurrence of *Salmonella* spp. and *Cryptosporidium* spp. in a French coastal watershed: relationship with fecal indicators. *FEMS Microbiol Lett* 218:203–209
- Lima MRP, Zandonade E, Sobrinho PA (2012) Characteristics of WWTP sludge after drying in greenhouse for agricultural purposes. *Water Sci Technol* 66:1460–1466
- Lindahl V, Bakken LR (1995) Evaluation of methods for extraction of bacteria from soil. *FEMS Microbiol Ecol* 16:135–142
- Lu Q, He ZL, Stoffela P (2012) Land application of biosolids in the USA: a review applied and environmental soil science. *Appl Environ Sci*. doi:10.1155/2012/201462
- Navarro I, Jiménez B, Cifuentes E, Lucario S (2009) Application of helminth ova infection dose curve to estimate the risks associated with biosolid application on soil. *J Water Health* 7:31–44
- Norma Oficial Mexicana México. (NOM-001-ECOL) (1996) Anexo I: Técnica para la determinación y cuantificación de huevos de helminto. <http://www.una.com.mx/content/sanidad/001ecol.pdf>. Accessed Nov 2013
- O'Connor GA, Elliott HA, Basta NT, Bastian RK, Pierzynski GM, Simse RC, Smith JE (2005) Sustainable land application. *J Environ Qual* 34:7–17
- Pinheiro IO, Castro MF, Mitterofhe A, Pires FAC, Abramo C, Ribeiro LC, Tibiriçá SHC, Coimbra ES (2011) Prevalence and risk factors for giardiasis and soil-transmitted helminthiasis in three municipalities of Southeastern Minas Gerais State, Brazil. *Parasitol Res* 108:1123–1130
- Redlinger T, Corela-Barud V, Graham J, Galindo A, Avitia R, Cardenas V (2002) Hyperendemic *Cryptosporidium* and *Giardia* in households lacking municipal sewer and water on the United States—México border. *Am J Trop Med Hyg* 66:794–798
- Rimhanen-Finne R, Vuorinen A, Marmo S, Malmberg GS, Hänninen ML (2004) Comparative analysis of *Cryptosporidium*, *Giardia* and indicator bacteria during sewage sludge hygienization in various composting process. *Lett Appl Microbiol* 38:301–305
- Robertson LJ, Hermansen L, Gjerde BK (2006) Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in Norway. *Appl Environ Microbiol* 72:5297–5303
- SANASA—Sanitation and Water Supplying Society (2011) <http://www.sanasa.com.br>. Accessed Nov 2013
- Santamaria J, Toranzos G (2003) Enteric pathogens and soil: a short review. *Int Microbiol* 6:5–9
- Searcy KE, Packman AI, Atwill ER, Harter T (2005) Association of *Cryptosporidium parvum* with suspended particles: impact on oocyst sedimentation. *Appl Environ Microbiol* 71:1072–1078
- Shamma M, Al-Adawi MA (2002) The morphological changes of *Ascaris lumbricoides* ova in sewage sludge water treated by gamma irradiation. *Radiat Phys Chem* 65:277–279
- Sidhu JPS, Toze SG (2009) Human pathogens and their indicators in biosolids: a literature review. *Environ Int* 35:187–201
- Silva PRP, Barbisan LF, Dagli MLZ, Saldiva PHN (2012) Sewage sludge does not induce genotoxicity and carcinogenesis. *Genet Mol Biol* 35:657–663
- Smith AL, Smith HV (1989) A comparison of fluorescein diacetate and propidium iodide staining and in vitro excystation for determining *Giardia intestinalis* cyst viability. *Parasitology* 99:329–331
- Thiriat L, Bigot V, Schwartzbrod J (1997) Evaluation of a procedure for detection of viable *Giardia* cysts in wastewater sludge. *Water Sci Technol* 35:377–380
- Udeh PJ, John G, Veenstra JN (2003) Field inactivation of oocysts exposed to agricultural land. *Water Air Soil Poll* 142:211–228
- United States Environmental Protection Agency (USEPA) (1999) Environmental regulations and technology: control of pathogens and vector attraction in sewage sludge. Revised in October 2011. Washington, DC. Report no. EPA/625/R-92/013
- United States Environmental Protection Agency (USEPA) (2005) Office of Water. Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. EPA 815-R-05-002. US Environmental Protection Agency. Washington DC, EEUU
- Victorica J, Galvan M (2003) Preliminary testing of a rapid coupled methodology for quantification/viability determination of helminth eggs in raw and treated wastewater. *Water Res* 37:1278–1287
- WHO—World Health Organization (2006) The International Decade for Action Water for Life—2005–2015. http://www.who.int/entity/water_sanitation_health/wwd7_water_scarcity_final_rev_1.pdf. Accessed Oct 2013
- Widmer G, Clancy T, Honorine DW, Miller D, Batzer GM, Pearson CB, Bukhari Z (2002) Structural and biochemical alterations in *Giardia lamblia* cysts exposed to ozone. *J Parasitol* 88:1100–1106