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Prevalence of *Cryptosporidium* oocysts and *Giardia* cysts in raw and treated sewage sludges

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Abstract

Treated sludge from wastewater treatment plants is commonly used in agriculture as fertilizers and to amend soils. The most significant health hazard for sewage sludge relates to the wide range of pathogenic microorganisms such as protozoa parasites.The objective of this study was to collect quantitative data on *Cryptosporidium* oocysts and *Giardia* cysts in the treated sludge in wastewater treatment facilities in Spain.

Sludge from five wastewater treatment plants (WWTPs) with different stabilization processes, has been analyzed for the presence of *Cryptosporidium* and *Giardia* in the raw sludge and after the sludge treatment. A composting plant (CP) has also been assessed.

After a sedimentation step, sludge samples were processed and (oo)cysts isolated by immunomagnetic separation (IMS) and detected by immunofluorescence assay (IFA).

Results obtained in this study showed that *Cryptosporidium* oocysts and *Giardia* cysts were present in 26 of the 30 samples (86.6%) of raw sludge samples. In treated sludge samples, (oo)cysts have been observed in all WWTP's analyzed (25 samples) with different stabilisation treatment (83.3%). Only in samples from composting plant no (oo)cysts were detected. This study provides evidence that (oo)cysts are present in sewage sludge-end products from wastewater treatment processes with the negative consequences for public health

Keywords: Cryptosporidium, *Giardia*, prevalence, sewage sludge, biosolids

Introduction

Cryptosporidium and *Giardia* are infectious protozoan parasites capable of causing gastrointestinal illnesses in both animals and humans. An infected person can shed up to $3x10^9$ oocysts [1] or up to $5x10^8$ cysts over the course of infection.[2] In fact, these parasites are the most common food and waterborne protozoa that affect humans and a wide range of animals, which can shed large numbers of the transmissive stages of these enteropathogens, (oo)cysts, in their surroundings.[3-7] Livestock operations and irrigation with polluted water can lead to the contamination of soil and vegetables with zoonotic faecal pathogens, in which *Giardia duodenalis* cysts and *Cryptosporidium parvum* oocysts are included.[8-11] *Cryptosporidium* and *Giardia* (oo)cysts are environmental resistant stages, and can survive at different temperatures and conditions.[12]

Aerobic wastewater treatment plants use sludge activated sludge process to reduce water pollution and the presence of microorganisms. Many of these microorganisms are trapped in, or adsorbed to particulates and are concentrated in the sludge. For example, during the separation of solid material from wastewater in both primary (settling) and secondary treatments, numerous pathogens remain associated with particulates thus concentrating them in the sludge.[13-14] Therefore, sludge can contain many of these parasites.[15-18]

Sewage sludge is the solid, organic material which remains after wastewater is treated and discharged from a wastewater treatment plant. Sludge is treated to stabilize the organic matter and reduce the amount of human pathogens.[19] Several treatments can be employed to reduce the pathogen content in sewage sludge, such as heat-drying, addition of hydrated lime (calcium hydroxide) to increase pH, composting, and biological digestion.[13] Biological digestion is the treatment method most frequently used to achieve stabilization, and involves decomposition of organic matter by microorganisms either in the presence of oxygen (aerobic digestion) or in its absence (anaerobic digestion).

Applications of sewage sludge-end products are an ecologically important means for the utilization of nitrogen and phosphorus. However, spreading sludge on agricultural lands, which has increased during the last years due to economic and environmental reasons,[20] facilitates circulation of *Cryptosporidium* and *Giardia* in the environment. It also contaminates shallow aquifers, potable waters [21] and food

cropped from land, thus posing a threat to human and animal health. During rainfall, (oo)cysts may end in surface and groundwater.[3] The very low infective dose of these parasites also suggests a health risk for workers exposed to sewage.[22]

Various studies have identified high levels of *Cryptosporidium* oocysts and *Giardia* cysts in both treated and untreated sewage sludge. [17-20,23-27] However, information on the effectiveness of sludge treatments on the inactivation of *Cryptosporidium a*nd *Giardia* is incomplete.[20,28-30] Consequently, it is important to quantify the risks of contamination associated with this practice, which in turn requires accurate methods to quantify the levels of *Cryptosporidium* and *Giardia* in biosolids, such as sewage sludge.

Several methods have been described to enumerate *Cryptosporidium* oocysts and *Giardia* cysts in different biosolids. These methods include ECP (ether clarification procedure), [31] NaCl flotation and differential density centrifugation with percoll-sucrose [32,33] and immunomagnetic separation (IMS) followed by immunofluorescence assay (IFA).[34-36,18] Recovery efficiencies associated with these methods have been determined by a number of researchers, but the results exhibit a considerable degree of variation.[37] A sedimentation step before immunomagnetic separation has been established by Massanet-Nicolau.[37] This method was used to recover stained cysts and oocysts (spiked organisms) from primary settled sewage sludge, anaerobically digested sewage sludge, and bovine manure.

The aim of this study was to determine the occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in raw and treated sludge from five wastewater treatment plants and from a composting plant to evaluate the levels of (oo)cysts present after each stabilization treatment .

Materials and Methods

Sludge samples and sampling sites

A total of 60 sludge samples (30 raw sludge and 30 treated sludge) have been processed from five wastewater treatment plants (WWTP) located in Eastern Spain: (WWTP1 ,WWTP2, WWTP3 ,WWTP4, WWTP5), that served different population (between 20,000 and 200,000 equivalent inhabitants) with different sludge treatments (Table 1) and which also received both agricultural and industrial inputs. Moreover, five samples from a composting plant (CP), which received sludge from some WWTPs, have also

been evaluated. In the case of the composting plant, dewatered sludge (raw) from different municipal sewage treatment plants is sent to the thermophilic composting process. Degradation occurs through longitudinal windrows (covered with a Gore cover). Ambient air is sucked through the piles during 10 days, where temperature is approximately 55ºC. The final product (from now on, referred to as compost) is stored for 30 days.

Samples were collected monthly over five samplings, from March to July in WWTP1, WWTP2 and WWTP3, and from September to January in WWTP4, WWTP5 and WWTP6. Each sample was transported to the laboratory, kept at 4ºC and tested within the next 12 h.

Cysts and oocysts detection in sludge

A reported method by Massanet-Nicolau [37] for sludge using a sedimentation step followed of inmunomagnetic separation has been used. One-gram samples of the sludge were deposited in 50-ml plastic centrifuge tubes (Corning). 25-ml volume of 0.1 M phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO) was added to the centrifuge tube containing the sludge sample and vortexed for 60 s. Another 25 ml of phosphate-buffered saline was then added to the sample, and the tube was inverted five times. The sample was left to stand for 60 min at room temperature, during which the heavier particles of debris settled at the bottom of the tube. At the end of the 60-min period, a 10-ml disposable pipette was used to transfer the top 45 ml of liquid to a clean 50-ml centrifuge tube. During this step, care was taken not to disturb the accumulated solids at the bottom of the tube. The 45 ml of liquid was then brought to a final volume of 50 ml with filtered deionized water and centrifuged for 5 min at 1,050 × *g* (with no braking during the deceleration phase). The rest of the analytical procedure was based on the U.S. Environmental Protection Agency Method 1623.[34] The sample was then further purified by immunomagnetic separation. After centrifugation, the pellet was resuspended in 10 ml of water in a Leighton tube, and immunomagnetic separation (IMS) was conducted using the commercially available GC Combo kit (Invitrogen-Dynal AS Oslo) according to the manufacturer's instructions. The final concentrate from the IMS was transferred onto the well of a slide and dried overnight at room temperature. The slide was labeled with fluorescent monoclonal antibodies fluorescein isothiocyanate (FITC) for *Giardia a*nd *Cryptosporidium* immunofluorescence assay

(IFA), according to the manufacturer's protocol, (Meridian Diagnostics, Inc., Cincinnati, OH).

Fifty microliters of (4,6-diamidino-2-phenylindole dihydrochloride) DAPI (Sigma-Aldrich, St. Louis, MO) solution (0.4 mg/ml in PBS) were placed in each well of the slide and allowed to stand at room temperature for 15 min; excess of DAPI solution was removed by washing the slides twice in PBS. Slides were placed under darkness, mounted and examined at 600X magnification using epifluorescence microscopy (Olympus BX 50, Tokyo, Japan). A blue filter (excitation, 480 nm; emission, 520 nm) was used to detect fluorescein isothiocyanate–conjugated MAb-labeled (oo)cysts, and a UV filter block was used for DAPI (excitation, 350 nm; emission , 450 nm).

Results

The occurrence of (oo)cysts has been studied in sludge from wastewater treatment plants before and after different stabilization treatments.

Data showed (Table 2) that oocysts of *Cryptosporidium* and cysts of *Giardia* were present in 26 of the 30 raw sludge samples (86.6%). The average concentration of *Cryptosporidium* was higher than *Giardia*'s average in WWTP1, WWTP2, WWTP3 and WWTP4 and lower in the raw sludge arriving to Composting Plant (CP). In WWTP5 same average of oocysts than cysts was found.

In the treated sludges, (oo)cysts were detected in WWTP1, WWTP2, WWTP3, WWTP4, and WWTP5 (83.3%). Only in the sludge from CP that was composted, no (oo)cysts were detected.

In WWTP2, WWTP4 (aerobic digestion) and WWTP5 (lime stabilization and heat drying) the average of oo(cysts) detected in treated sludge was higher than in sludge before treatment. In WWTP1 and WWTP3, the average of (oo)cysts was lower in the sludge after treatment (anaerobic digestion and lime stabilization respectively) but in some samples, (oo)cysts counts were higher in treated sludge than in mixture sludge before treatment, particularly in WWTP1 (Table 2). Maximum levels of *Cryptosporidium* of 498 oocysts/g and 248 cysts/g of *Giardia* have been found in WWTP1 during the sampling 1 (Table 2), although the average of (oo)cysts was lower in treated sludge than in raw sludge. Sludge from this WWTP is used in agriculture,

which may pose a health risk. In the CP, counts of (oo)cysts after composting were 0 in all samplings (Table 2).

Discussion

The sedimentation step followed by IMS –IFA [37] used in this work has proven to be useful for simultaneous detection of C*ryptosporidium* oocysts and *Giardia* cysts in raw and treated sludge. Recovery efficiencies associated with this method were approximately 40 to 60% and were significantly greater than those associated with similar methods based on sucrose flotation ($P< 0.001$).[37] Other authors [35] also found high counts of *Cryptosporidium* oocysts, 70%, using IMS and IFA after a spontaneous sedimentation. In contrast, Orlofsky et al. [36] obtained better results avoiding the sedimentation step.

In raw sludge samples, higher counts of *Cryptosporidium* oocysts than *Giardia* cysts have been detected (Table 2). Similar findings were reported in a study in Ireland.[27]

The different treatments used for sludge stabilisation in the studied WWTPs (Table 1) have not proven to be effective for total (oo)cysts elimination. Among the sludge treatments studied in this work, aerobic digestion and lime stabilisation showed higher average concentration of (oo)cysts in treated sludge than in raw sludge.

Furthermore, *Cryptosporidium* oocysts counts in treated sludge were higher than *Giardia* cysts' counts in WWTP1, WWTP2, WWTP3 and WWTP4. Only in WWTP5, higher counts of cysts than oocysts were found. Hence, *Cryptosporidium* oocysts appear to be more resistant to treatments compared to *Giardia* cysts.

Of the different disposal methods available for biosolids, composting presents certain advantages over more traditional methods, such as direct land application, incineration or burying in landfills. Composting is known to decrease the load of human pathogenic microorganisms potentially present in biosolids. It also yields an end product rich in organic matter and nutrients that can be used as a soil supplement for different environmental practices. In our study, only in composted sludge, no (oo)cysts were found. Other authors [38,39] agree that *Giardia* and *Cryptosporidium* are relatively sensitive to heat treatment and are rapidly reduced within thermophilic composting. In contrast, in a study about occurrence of pathogens in treated sludge,[40] *Cryptosporidium* oocysts were still detected in most compost samples. The inactivation

of *Giardia* and *Cryptosporidium* (oo)cysts during composting of biosolids was monitored by Rimhanen-Finne et al. [20] and results showed that 44% and 37.5% of compost samples were positive for cysts and oocysts respectively after 10 weeks of composting.

Anaerobically digested biosolids have been reported to contain up to 10^1oocysts/g of *Cryptosporidium* and 10^2cysts/g *Giardia*. [15] In the dewatering processes, 50% percent of the pathogens were assumed to attach to sludge particulates. High *Giardia* cyst numbers (10^2cysts/g) following anaerobic digestion were reported in a study in Perth (Australia). [40] Other authors, [41,42] have also found *Giardia* cysts in both digested and undigested sludge, and there were no significant differences between their occurrence in raw and treated sludge, concluding that *Giardia* cysts did not appear to be reduced by anaerobic digestion. In our study, the anaerobically digested sludge from WWTP1 also contained up to 10^2/g (oo)cysts. It is possible that during the sludge treatment, such as anaerobic digestion where temperature is generally more than 35ºC, (oo)cysts might lose viability and infectivity rapidly. In most of the studies, [15,17,20] viability assessment of oo(cysts) is either not carried out or was inconclusive.

Regarding aerobic digestion, results obtained in our study in both WWTP2 and WWTP4, showed that the average of (oo)cysts present in sludge after stabilization were higher than in raw sludge. In a study about efficiency of a wastewater treatment plant for removal pathogens in Spain, [43] *Giardia* was detected in the sludge after aerobic digestion and no *Cryptosporidium* was detected; other authors [15] found higher reductions in *Cryptosporidium* (2.96 log) compared to *Giardia*'s (1.40 log) during aerobic digestion of sludge.

In the present study, higher counts of *Cryptosporidium* oocysts than *Giardia* cysts have been detected in treated sludge from wastewater treatment plants, where sludge treatment was either lime stabilization or anaerobic or aerobic digestion. However, some authors [25] found that quicklime stabilization treatments reduced the load of both pathogens to non-detectable levels. Other studies, [29] have reported cysts of *Giardia* in sludge after lime stabilization, but no *Cryptosporidium* oocysts were detected. Similar findings reported in sewage sludge [31,44] have been explained by much higher relative incidence of giardiasis than cryptosporidiosis cases, that is, 300 vs. 10, respectively, in communities served byWWTPs.[29] In a study in Poland [25] it was found that the mean concentration of *Cryptosporidium* oocysts in sewage sludge

samples in two wastewater treatment plants was significantly lower than the concentration of *Giardia* cysts found in these samples. *Giardia* cysts are more frequently isolated in higher numbers throughout the year from wastewater and biosolids than *Cryptosporidium* oocysts, which showed seasonal variation. [45,46]

Limited information is available on the fate of protozoan pathogens in biosolids. Moreover, interpretation and comparison of data is difficult due to inconsistencies in sampling, concentration and recovery procedure. Landfill leachate and sewage sludge contained high numbers of potentially viable, human-virulent species of *Cryptosporidium* and *Giardia.* [25] Due to the massive amounts of sewage sludge used by agriculture or deposited in landfills [21,47-49] and the environmental robustness of these pathogens, [50-53] environmental contamination derived from landfill leachates and sewage sludge presents serious public and veterinary threats. For the sewage sludge-end products to be used without restriction on agricultural lands to grow ready-to-eat crops, the pathogen load must be reduced to a level which does not pose acceptable public health or veterinary risks.[48,49]

Conclusions

Sludge treatments such as anaerobic and aerobic digestion, lime stabilization and heat drying do not eliminate *Cryptosporidium* oocysts and G*iardia* cysts.

Composting appears to be an interesting alternative to traditional disposal methods, since it is the only stabilization treatment which eliminates *Giardia* and *Cryptosporidium* (oo)cysts.

More information on the occurrence and levels of pathogens and indicators in biosolids is necessary to characterize and anticipate the risks associated with treated sewage sludge. An evaluation of the risks associated with the disposal or use of biosolids and proper regulations are both necessary from a public health perspective.

Further research is needed to establish whether treatment options could render the sewage sludge into pathogen-free biosolids, which can be safely disposed into the environment.

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Disclosure statement

No potential conflict of interest was reported by the author(s)

References

[1] Okhuysen PC, Chappell L, Crabb JH, et al. Virulence of three distinct *Cryptosporidium parvum* isolates for healthy adults. J Infect Dis. 1999;180:1275–1281.

[2] Medema GJ, Schijven JF. Modeling the sewage discharge and dispersion of *Cryptosporidium* and *Giardia* in surface water. Wat Res. 2001;35:4307–4316

[3] Slifko TR, Smith HV, Rose JB. Emerging parasite zoonoses associated with water and food .Int J Parasitol. 2000;30:1379-1393

[4] Thompson RCA. Giardiasis as re-emerging infectious disease and its zoonotic potential. Int J Parasitol. 2000;30:1259-1267

[5] Smith HV, Caccio SM, Cook N et al. *Cryptosporidium* and *Giardia* as foodborne zoonoses. Vet Parasitol. 2007;149: 29–40

[6] Ongerth JE. The concentration of *Cryptosporidium* and *Giardia* in water . The role and importance of recovery efficiency. Wat Res. 2013;47:2479 -2488.

[7] Ryan U, Caccio SM. Zoonotic potential of *Giardia*. Int J Parasitol. 2013;43:943-956.

[8] Bicudo JR, Goyal SM. Pathogens and manure management systems: a review. Envirol Technol. 2003;24:115-130.

[9] Kim SB, Corapcioglu MY. Vertical Transport of *Cryptosporidium parvum* oocysts through sediments. Environ Technol. 2002;23:1435-1446.

[10] Amorós I, Alonso JL, Cuesta G. *Cryptosporidium* oocysts and *Giardia* cysts on salad products irrigated with contaminated water. J Food Prot. 2010;73:1138–1140.

[11] Liang Z, Keeley A. Detection of viable *Cryptosporidium parvum* in soil by reverse transcription–Real-Time PCR Targeting hsp70 mRNA. Appl Env Microbiol. 2011;77:6476–6485.

[12] Fayer R, Trout J, Jenkins MC. Infectivity of *Cryptosporidium parvum* oocysts in seawater and Eastern oysters (*Crassostrea virginica*) in the Chesapeake Bay. Appl Environ Microbiol. 1998;64:1070–1074.

[13] Godfree A, Farrell J. Processes for managing pathogens. J Environ Qual. 2005;34: 105-113.

[14] Smith Jr JE, Surampalli RY, Reimers RS, et al. Disinfection processes and stability refinements to biosolids treatment technologies. Pract period hazard, toxic, radioact waste manag. 2008; 12: 10–17.

[15] Chauret C, Springthorpe S, Sattar S. Fate of *Cryptosporidium* oocysts, *Giardia* cysts, and microbial indicators during wastewater treatment and anaerobic sludge digestion. Can J Microbiol. 1999;45:257–262.

[16] Romdhana MH, Lecomte, D Ladevie B, et al. Monitoring of pathogenic microorganisms contamination during heat drying process of sewage sludge. Process Safety and Environ Protection. 2009;87:377–386.

[17] Sidhu JPS, Toze SG. Human pathogens and their indicators in biosolids: A literature review. Environ Int. 2009;35:187–201.

[18] Khouja LBA, Cama V, Xiao L. Parasitic contamination in wastewater and sludge samples in Tunisia using three different detection techniques. Parasitol Res. 2010;107:109–116.

[19] van Frankenhuyzen JK, Trevors JT, Lee H, et al. Molecular pathogen detection in biosolids with a focus on quantitative PCR using propidium monoazide for viable cell enumeration. J Microbiol Methods. 2011;87:263–272.

[20] Rimhanen-Finne R, Vourinen A, Marmo S, et al. Comparative analysis of *Cryptosporidium* and *Giardia* andindicator bacteria during sewage sludge hygienization in various composting processes. Lett Appl Microbiol. 2004;38:301–305.

[21] Straub TM, Pepper IL, Gerba CP. Hazards from pathogenic microorganisms in land-disposed sewage sludge. Rev Environ Contam Toxicol. 1993;132:55-91.

[22] Schlosser O, Grall D, Laurenceau MN. Intestinal parasite carriage in workers exposed to sewage. Eur J Epidemiol. 1999;15:261-265.

[23] Nordic Council of Ministers, 2006 © Copenhagen. Protozoan Parasites in Sewage Sludge. TemaNord. 2006;596.

[24] Graczyk TK, Lucy FE, Tamang L, et al. Human enteropathogen load in activated sewage sludge and corresponding sewage sludge-end products. Appl. Environ Microbiol. 2007a;73:2013–2015.

[25] Graczyk TK, Kacprzak M, Neczaj E, et al. Occurrence of *Cryptosporidium* and *Giardia* in sewage sludge and solid waste landfill leachate and quantitative comparative analysis of sanitization treatments on pathogen inactivation. Environmental Res. 2008;106: 27–33.

[26] Guzmán C, Jofre J, Montemayor M, et al. Occurrence and levels of indicators and selected pathogens in different sludges and biosolids. J. Appl. Microbiol. 2007;103:2420–2429.

[27] Cheng HW, Lucy FE, Graczyk TK, et al. Fate of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and *Giardia duodenalis* cysts during secondary wastewater treatments. Parasitol Res. 2009;105:689–696.

[28] Ashokkumar M,Vu T, Grieser F, et al. Ultrasonic treatment of *Cryptosporidium* oocysts. Water Sci Technol. 2003;47:173–177.

[29] Rimhanen-Finne R, Ronkainen P, Hanninen ML. Simultaneous detection of *Cryptosporidium parvum* and *Giardia* in sewage sludge by IC-PCR. J Appl Microbiol. 2001; 91: 1030–1035.

[30] Collins MV, Flick GJ, Smith S, et al. The effect of E-beam irradiation and microwave energy on Eastern oysters *(Crasssostrea virginica*) experimentally infected with *Cryptosporidium parvum*. J Eukaryot Microbiol. 2005;52:484–488.

[31] Santos LU, Bonatti TR, Cantusio R, et al. Occurrence of *Giardia* cysts and *Cryptosporidium* oocysts in activated sludge samples in Campinas, SP, Brazil. Rev Inst Med S Paulo. 2004;46:309–313.

[32] Bukhari Z, Smith HV, Sykes N, et al. Occurrence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in sewage effluents and effluents from treatment plants in England. Water Sci Technol. 1997;35:385–390.

[33] Kuczynska E, Shelton DR. Method for detection and enumeration of *Cryptosporidium parvum* oocysts in feces, manures and soils. Appl Environ Microbiol. 1999;65:2820-2826.

[34] U.S. Environmental Protection Agency (USEPA). Method 1623: *Cryptosporidium* and *Giardia* in water by IFA/IMS/FA. EPA 815-R-05-002. Washington, DC, USA: Office of Water, United States Environmental Protection Agency; 2005.

[35] Iacovski RB, Barardi CRM, Oliveira CM. Detection and enumeration of *Cryptosporidium* sp. oocysts in sewage sludge samples from the city of Florianópolis (Brazil) by using immunomagnetic separation combined with indirect immunofluorescence assay. Waste Manage Res. 2004;22:171–176.

[36] Orlofsky E, Gillor O, Melli A, et al. Simultaneous detection of *Giardia lamblia* and *Cryptosporidium parvum* (oo)cysts in soil using immunomagnetic separation and direct fluorescent antibody staining .J Microbiol Methods. 2013;94:375–377.

[37] Massanet-Nicolau J. New Method Using Sedimentation and Immunomagnetic Separation for Isolation and Enumeration of *Cryptosporidium parvum* Oocysts and *Giardia lamblia* cysts. Appl Environ Microbiol. 2003;69:6758–6761.

[38] Feachem RG, Bradley DJ, Garlick H, et al. Sanitation and Disease: Health Aspects of Excreta and Wastewater Management. 1983 New York, NY: John Wiley and Sons.

[39] Wichuk KM, McCartney D. A review of the effectiveness of current time temperature regulations on pathogen inactivation during composting. Journal of Environmental Engineering Science. 2007;6:573-586.

[40] Hu CJ, Gibbs RA, Mort NR, et al. *Giardia* and its implications for sludge disposal. Water Sci Technol. 1996;34:179–186.

[41] Soares AC, Straub TM, Pepper IL, et al. Effect of anaerobic digestion on the occurrence of enteroviruses and *Giardia* cysts in sewage sludge. J Environ Sci Health. 1994;29:1887–97.

[42] Sykora JL, Sorber CA, Jakubowski W, et al. Distribution of *Giardia* cysts in wastewater. Wat Sci. Tech. 1991; 24:187-192.

[43] Marín I, Goñi P, Lasheras AM, et al. Efficiency of a Spanish wastewater treatment plant for removal potentially pathogens: Characterization of bacteria and protozoa along water and sludge treatment lines. Ecol Eng. 2014; 74:28–32.

[44] Robertson LJ, Paton CA, Campbell AT, et al. Giardia cysts and *Cryptosporidium* oocysts at sewage treatment works in Scotland, UK. Water Res. 2000;34:2310–2322.

[45] Payment P, Plante R, Cejka P. Removal of indicator bacteria, human enteric viruses, *Giardia* cysts, and *Cryptosporidium* oocysts at a large wastewater primary treatment facility. Can J Microbiol. 2001;47:188-93.

[46] Caccio SM, De Giacomo M, Aucilino FA, et al. 2003. *Giardia* cysts in wastewater treatment plants in Italy. Appl Environ Microbiol. 2003;69:3393–3398.

[47] Szostakowska B, Kruminis-Lozowska W, Racewicz M, et al. *Cryptosporidium* parvum and *Giardia* lamblia recovered from flies on a cattle farm and in a landfill. Appl Environ Microbiol. 2004;70:3742–3744.

[48] Westrell T, Schonning C, Stenstrom TA, et al. QMRA (quantitative microbial risk assessment) and HACCP (hazard analysis and critical control points) for management of pathogens in wastewater and sewage sludge treatment and reuse. Water Sci Technol. 2004;50:23–30.

[49] Gale P. Land application of treated sewage sludge: quantifying pathogen risk from consumption of crops. J Appl Microbiol. 2005;98:380–396.

[50] deRegnier DP, Cole L, Schupp DG, et al. Viability of *Giardia* cysts suspended in lake, river, and tap water. Appl Environ Microbiol. 1989;55:122–129.

[51] Tamburrini A, Pozio E. Long-term survival of *Cryptosporidium parvum* oocysts in seawater and in experimentally infected mussels *(Mytilus galloprovincialis*). Int J Parasitol. 1999;29:711–715.

[52] Hutchinson ML, Walters LD, Moore T, et al. Fate of pathogens present in livestock wastes spread onto fescue plots. Appl Environ Microbiol. 2005a;71:691–696.

[53] Hutchinson ML, Walters LD, Moore T, et al. Decline of zoonotic agents in liquid livestock wastes stored in batches on-farm. Lett Appl Microbiol. 2005b;99:58–65.

Table 1: Characteristics of wastewater treatment plants: population served and different sludge treatments

Wastewater Treatment Plant	Population equivalent	Secondary treatment	Tertiary treatment	Sludge Treatment
WWTP1	205650	Activated sludge and sedimentation	Sand filtration, ultraviolet	Anaerobic Digestion
WWTP2	49702	Activated sludge and sedimentation	None	Aerobic Digestion
WWTP3	21255	Activated sludge and sedimentation	Sand filtration, ultraviolet	Lime stabilization
WWTP4	20055	Activated sludge and sedimentation	None	Aerobic Digestion
WWTP5	20092	Activated sludge and sedimentation	Ultraviolet	Lime stabilization and heat drying
CP[a]	-	-	-	Composting

[a]Composting plant

1 Table 2: *Cryptosporidium* oocysts and *Giardia* cysts in raw and treated sludge

	WTP 1				WTP 2				WTP 3				WTP 4				WTP 5				CP			
	Oocysts/g		Cysts/g		Oocysts/g		Cysts/g		Oocysts/g		Cysts/g		Oocysts/g		Cysts/g		Oocysts/g		Cysts/g		Oocysts/g		Cysts/g	
Sampling	raw sludge	treated sludge	raw sludge	treated sludge	raw sludge	treated sludge	raw sludge	treated sludge	raw sludge	treated sludge	raw sludge	treated sludge	raw sludge	treated sludge	raw sludge	treated sludge	raw sludge	treated sludge	raw sludge	treated sludge	raw sludge	treated sludge	raw sludge	treated sludge
1	354	498	124	248	21	69	4	4	268	48	85	75	0	55	0	8	0	5	0	1	14	0	1	0
2	140	87	174	83	13	24	3	1	164	125	74	93	0	21	1	31	0	8	1	24	51	0	71	0
3	325	173	213	182	18	18	9	20	171	79	171	27	11	19	6	36	12	8	4	27	48	0	98	0
4	126	104	97	48	2	8	3	3	39	48	30	76	7	25	1	14	5	5	18	58	27	0	63	0
5	59	100	77	100	2	7	2	1	52	17	155	13	5	12	0	22	6	7	0	2	22	0	64	0
Average	201	192	137	132	11	25	4	6	139	63	103	57	5	26	2	22	5	7	5	22	32	0	59	0
SD	131	174	56	81	9	25	3	8	95	41	59	35	5	17	3	12	5	2	8	23	16	0	36	0
*uncertainty	59	78	25	36	4	11	1	4	42	18	26	16	2	8	1	5	2	1	4	10	7	0	16	0

*SD/ total sampling