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Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in effluent from sewage treatment plant from eastern Poland

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Abstract

Cryptosporidium spp. and *Giardia lamblia* (synonyms: *Giardia duodenalis, Giardia intestinalis*) are emerging protozoa causing disease in humans and animals worldwide. These parasites can pose a serious threat to immunocompromised people, for whom the symptoms are more severe and may include abdominal pain, watery diarrhoea, nausea, headaches, malaise, and fever. One of the sources of these parasites can be treated wastewater from wastewater treatment plants (WTPs). Samples of treated wastewater (effluent), each of 10 L volume, were collected from 13 municipal WTPs located in eastern Poland. *Cryptosporidium* oocysts and *Giardia* cysts were separated by the immunomagnetic method. The presence and/or concentration of protozoan (oo)cysts in effluent samples were determined by direct immunofluorescent microscopy, nested PCR and Real Time PCR. Viability of (oo)cysts was determined by double-staining with the use of Live/Dead BacLight kit (Invitrogen). *Cryptosporidium* spp. oocysts were detected in 8 WTPs (61.5%) and *Giardia* spp. cysts in 11 WTPs (84.6%) by microscopic analysis. Both pathogens were detected in samples from 7 WTPs. Median concentrations of *Cryptosporidium* and *Giardia* (oo)cysts in 13 examined samples were 2.2/L and 6.6/L, respectively, while mean concentrations were 28.5/L and 113.6/L, respectively. In positive samples, *Cryptosporidium* oocysts concentrations ranged from 0.4 – 154.1 oocysts per litre, and *Giardia* cysts concentrations ranged from 0.7 – 660 cysts per litre. By nested PCR, *Giardia* DNA was detected in 4 samples of the 13 examined, (30.8%) while *Cryptosporidium* DNA was never detected. In Real Time PCR, positive results for *Giardia* were obtained in 5 samples (38.5%) and in none of the samples for *Cryptosporidium*, with the exception of one equivocal result. Viable (oo)cysts of *Cryptosporidium* and *Giardia* were detected in 3 out of 4 samples examined, in the ranges of 12.5 – 60% and 50 – 100% of total (oo)cysts, respectively. In view of our preliminary study, the presence of oocysts and cysts (largely viable) in effluents from WTPs imply a risk of transmission of waterborne protozoan parasites to humans. Therefore, additional wastewater purification procedures are necessary.

Key words

sewage treatment plants, effluent, *Giardia*, *Cryptosporidium*, concentration, viability, eastern Poland

INTRODUCTION

Cryptosporidium spp. and *Giardia lamblia* (synonyms: *Giardia duodenalis, Giardia intestinalis*) are emerging protozoa causing diarrheal disease in humans and animals worldwide [1, 2, 3]. According to Guy et al. [4] these parasites are major causes of waterborne enteric disease throughout the world. Currently, there are 13 recognized species of *Cryptosporidium* and 6 species of *Giardia* that can cause infections in humans or animals. Among them, *C. hominis*, *C. parvum* and *Giardia lamblia* are responsible for most human infections [5, 6]. *Giardia lamblia* is divided into at least 7 genetically distinct assemblages (A to G), differing with host specificity. Zoonotic potential have assemblages A (divided into 2 major subtypes, A-I and A-II) and B [7]. At least 325 outbreaks associated with waterborne transmission of protozoan parasitic agents have been reported [8]. *Giardia lamblia* and *Cryptosporidium parvum* have been responsible for the vast majority of those outbreaks (40.6% and 50.8%, respectively) [9]. These parasites are transmitted by the faecal-oral route through contaminated water and food. In 1993, an extremely large waterborne outbreak of cryptosporidiosis occurred in Milwaukee, Wisconsin, USA, which resulted in 403,000 people being infected and included at least 70 fatalities [10]. In Europe, a big waterborne outbreak of giardiasis with 1,300 laboratory-confirmed cases took place in Bergen, Norway [11].

The (oo)cysts are resistant to conventional routine disinfectants. The prevalence of *Giardia* infection in people was estimated at 2 – 5% in industrialized countries and 20 – 30% in developing countries [12].

Studies including analysis of sewage for the parasites *Cryptosporidium* and *Giardia*, have been performed throughout the world [3, 13]. *Cryptosporidium* spp. and *Giardia lamblia* have been detected in effluents and biosolids from wastewater treatment plants, but so far only a little is known about wastewater treatment and how it may affect their survival and removal [14, 15]. Many authors report a high reduction of proozoan (oo)cysts as a result of sewage treatment processes: by 96 – 100% [16, 17, 18, 19] or by 1.4 – 3.0 $\log_{10}$ [10, 20, 21].

Primary treatment involves the settlement of preliminary treated wastewater in a sedimentation tank, whereas in

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secondary treatment the wastewater is aerobically broken down during sludge activation in an aeration tank, oxidation ditch, or by a percolating filter system [22]. Following this treatment, the wastewater is removed to a clarifier where the solids settle by gravity. Raw liquid sludge, with more than 94% of water, is regularly removed from the bottom of either a primary sedimentation tank (in the case of primary sludge) or from a clarifier (in the case of secondary sludge), and the sewage sludge is transported to a sludge drying bed for thickening and dewatering [22]. Lim et al. [23] reported that the treatment of sewage with extended aeration is better than that in an aerated lagoon. Graczyk et al. [24] expressed an opinion that reduction of the concentration of *Cryptosporidium* and *Giardia* in dewatered and biologically stabilized sewage sludge is significantly greater compared to sludge activation process.

Depending on the efficiency of the conventional wastewater treatment plants, (oo)cysts may pass through the treatment process and contaminate surface waters (i.e. rivers, recreational waters) and agricultural lands, which leads to the potential infection of humans [9, 25]. Oocysts of *Cryptosporidium* were detected from a few to 100% of tested surface waters at concentrations ranging from 0.001 – 100/L [26], and from 9.5 – 22% of groundwater samples [1].

Activated sludge systems demonstrate average removal rates of 92% and 87% for parasite eggs and cysts, respectively, which indicates the possibility of survival of protozoan (oo)cysts and helminth eggs beyond typical retention times for treatment processes [27]. For this reason, there is a duty to monitor the safety of these substances, including the obligation for parasitological examination.

OBJECTIVE

The aim of the presented study was to estimate the prevalence of *Cryptosporidium* oocysts and *Giardia* cyst in wastewater (effluent) from selected sewage treatment plants located in eastern Poland from the aspect of surface waters contamination, and the potential threat to human health.

MATERIAL AND METHODS

Sampling. Samples of treated wastewater (effluent), each of 10 L volume, were collected from 13 municipal wastewater treatment plants (WTP) located in eastern Poland (Fig. 1). Details of the treatment process used in each of the WTPs are shown in Table 1. The samples were taken between September 2012 – February 2013, and stored at 4 °C before analysis.

Figure 1. Localization of examined waste treatment plants.

Table 1. Characteristics of studied wastewater treatment plants (WTP)

WPT codes	Type	Water region	Population of inhabitants supported by WTP	Flow capacity of WTP (m³/day)	Mass of dehydrated sewage sludge (kg/day)
A	M, PUB2	CW	20,000 (100%)	1,620	405
B	M, B	CW	2,158 (89.09%)	200	50
C	M, B	CW	6,274 (53.35%)	1,960	490
D	M, B	CW	2,448(69.9%)	905	226
E	M, B	UW	11,970 (99.9%)	2,500	625
F	M, PUB2	CW	10,295 (100%)	1,620	405
G	M, Non-B	CW	3,040 (75.96%)	2,200	550
H	M, B	CW	11,635 (87.83%)	3,200	800
I	M, PUB1	CW	63,260 (67.66%)	25,000	6,250
J	M, PUB2	CW	40,017 (89.09%)	9,000	2,250
K	M, PUB2	CW	15,310 (91.62%)	4,000	1,000
L	M, PUB2	CW	25,000 (12.5%)	1,500	500
M	M, PUB2	CW	15,921 (100%)	4,730	1,182

M – mechanical WTP; **B** – biological WTP fulfilling standards of effluent for agglomerations with <15,000 inhabitants; **Non-B** – biological WTP not fulfilling standards of effluent for agglomerations with <15,000 inhabitants; **PUB1** – biological WTP with increased removing compounds of nitrogen (N) and phosphorus (P) fulfilling standards of effluent for agglomerations with ≥ 100,000 inhabitants; **PUB2** – biological WTP with increased removing compounds of nitrogen (N) and phosphorus (P) fulfilling standards of effluent for agglomerations with ≥ 15,000 RLM < 100,000 inhabitants; **UW** – Upper Vistula Water Region; **CW** – Central Vistula Water Region

Immunomagnetic separation. The wastewater samples were centrifuged at 1,500 g for 10 min. The supernatant was aspirated and removed. Next, immunomagnetic separation (IMS) was performed using Dynabeads GC-Combo kit, (Invitrogen Dynal). The IMS procedure was performed according to the US EPA method 1623 [28]. From the pellets obtained after centrifugation, 0.5 ml was transferred to a Leighton tube, already containing 1 ml of 10 × SL buffer A and 1 ml of 10 × SL buffer B, and supplied with distilled water (DW) to 10 ml. After that, 100 µl of anti-*Cryptosporidium* and anti-*Giardia* beads were added to the tube, and the sample rotated for 1 h at room temperature. The tube was placed in a magnetic particle concentrator and gently rocked for 2 min. After removing the supernatant, and removal of the tubes from the magnetic particle concentrator, 1 ml of 1 × SL buffer A was added to each tube. The suspension of complexes of beads and (oo)cysts was transferred into a 1.5 ml Eppendorf tubes. The tubes were placed in another magnetic particle concentrator and rocked for 1 min. After removing supernatant and magnet, 50 µl of 0.1 N HCl was added, and the tube was vortexed for 15 s. Tubes were allowed to stand for 10 min in a vertical position. After that, the magnet was again inserted. The tubes were allowed to stand for 2 min to collect the beads at the back side of the tube. Next, suspension of released (oo)cysts was transferred to the next tube, containing 5 µl of 1N NaOH.

Direct Immunofluorescence Assay (DFA). DFA was performed using commercial test Aqua-Glo™ G/C Direct Comprehensive Kit (Waterborne Inc., USA). 15 microliters of IMS product were put on microscopic slide and allowed to

dry at room temperature. Next, samples were fixed by 50 µl of methanol and allowed to dry. After that, 50 µl of 4'6-diamidino-2-phenyl indole (DAPI) in PBS (0.4 µg DAPI/ml) was dropped and left for 4 min at room temperature. After removing DAPI, the slide was rinsed by adding 100 µl wash buffer and left for 1 minute. The wash buffer was removed, and 50 µl of conjugate anti-*Cryptosporidium*/*Giardia* with fluorescein isothiocyanate (FITC) placed in each well. The slides were placed in a humidified chamber and incubated at 37 °C for 30 min. Then, the washing step was performed (as described above). One drop of BlockOut™ counterstain (to reduce non-specific background fluorescence) was added to each well, and the slide incubated for 1 minute at room temperature. After washing (as described above) and drying the slide, a drop of Fade™ mounting medium was placed in each well, covered by glass, and viewed under an epifluorescence microscope (× 400). *Cryptosporidium* and *Giardia* (oo) cysts were identified on the basis of their size, shape, and structure, according to guideline described in method 1623 [28]. Positive and negative controls were used.

The number of pathogens per litre of effluent was counted by multiplication of the number of parasites detected microscopically in 15 µl of product IMS by 3.67 (factor for whole volume of IMS product), multiplication of 0.5 ml (volume used in IMS) by total volume of pellet obtained after centrifugation of sample, and final division by 10.

Evaluation of viability. Viable cysts of *Giardia lamblia*, produced by the passage of human strain H3 (assemblage B) through Mongolian gerbils, were obtained (Waterborne Inc., New Orleans, LA, USA). The cysts were suspended in phosphate-buffered saline with penicillin, gentamicin and Tween 20 and were stored at 4 °C until use.

The IOVA strain of live *Cryptosporidium parvum* oocysts produced by passage in calves were obtained from Waterborne Inc., USA. Oocysts were suspended in phosphate-buffered saline (PBS) containing antibiotics and stored at 4 °C until use.

The number of (oo)cysts in the stock solution was determined in the laboratory by using a Fuchs-Rosenthal chamber. Thus, precise numbers of cysts were determined.

Inactivation of control (oo)cysts was performed by treatment at 70 °C in a water bath for 30 min. Dead and live (oo)cysts were used as controls in the experiment.

Double-staining of (oo)cysts with the use of Live/Dead BacLight kit (Invitrogen) was performed according to Taghi-Kilani et al. [29]. After thawing of the kit at room temperature in light protected conditions, equal volumes of reagents A and B were combined in a 1.5 ml Eppendorf tube and mixed. The tube was wrapped with aluminum foil. 3 microliters of this mixture was then added to 97 µl of DW, containing 1×10^6 *Cryptosporidium* oocysts and a similar amount of *Giardia* cysts.

After incubation at 37 °C for 60 min, 15 µl of aliquots were placed on a slide and microscopical observations were made. Preparations were observed under a fluorescence microscope, using a 40 × magnification and filter for wave length 470 nm (excitation) and 490 nm (emission). Colour and intensity of live (dark green) and dead (light green or orange/yellow) (oo)cysts staining were visually assessed. The number of unstained (oo)cysts was also assessed.

In order to assess viability of (oo)cysts isolated from effluent samples collected in 4 selected WTPs, the same procedure was performed.

Nested PCR. The remaining IMS product (40 µl) was designed for the isolation DNA with the use of QIAamp DNA Mini kit (QIAGEN GmbH, Germany), according to manufacturer's instructions with minor modification: samples underwent seven freeze-thawing cycles by placing the tubes in liquid nitrogen, followed by immediately placing the tubes in 70 °C water. Next, samples with 20 µl of proteinase K and 180 µl of ATL buffer were incubated overnight at 56 °C. The extracted DNA was stored at –20 °C until PCR assay.

For molecular identification of *Giardia* spp., a semi-nested PCR was performed according to Caccio et al. [30] with slight modifications. Each reaction mixture (50 µl) consisted of 1 × buffer and contained 3.2 mM of $MgCl_2$, 0.1 µM of each primer, 1.3 units of Taq polymerase (Qiagen), 0.2 mM of each dNTP's (Fermentas), and 2.5 µl of DNA. Amplification was carried out as follows: an initial cycle at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s. Final extension was carried out at 72 °C for 7 min. Each semi-nested PCR product was subjected to electrophoresis on 1.5% agarose gel, and stained with ethidium bromide.

Molecular identification of *Cryptosporidium* spp. was performed on the basis of 18S SSU rRNA gene analysis using the primers previously described by Xiao et al. [31] in modification by Santin et al. [32].

Negative and positive DNA controls were included in each of PCR reactions. Amplification was performed using a TProfessional 48 thermal cycler (Biometra GmbH, Göttingen, Germany). After conducting the electrophoresis, PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide under UV light.

Real Time PCR. To amplify a 74-bp fragment of β-giardin gene of *Giardia* and 151 bp fragment of COWP gene of *Cryptosporidium,* Real Time PCR according to Guy and al. [4] was performed with some modifications: the commercial mix IQ Supermix (Bio-Rad) was used and number of cycles increased to 50. Real Time PCR was performed in a CFX96 Bio-Rad thermocycler.

RESULTS

Prevalence of *Giardia* and *Cryptosporidium* oocysts determined by microscopy (DFA). *Cryptosporidium* spp. oocysts were detected in 8 WTPs (61.5%) and *Giardia* spp. cysts in 11 WTPs (84.6%) by microscopic analysis. Both pathogens were detected in samples from 7 WTPs. In positive samples, *Cryptosporidium* oocysts concentrations ranged from 0.4 – 154.1 oocysts per litre, and *Giardia* cysts concentrations ranged from 0.7 – 660 cysts per litre. The highest number of *Cryptosporidium* oocysts was detected in sample from WTP L (154.1 oocysts per litre), the lowest in the sample from WTP C (0.4 oocysts per litre) (Tab. 2). The highest number of *Giardia* cysts was detected in the sample from WTP G (660 cysts per litre), and the lowest in the sample from WTP B (0.7 cysts per litre).

Evaluation of viability. The results of Live/Dead BacLight staining of 4 selected samples showed viable (green or dark green colour) *Cryptosporidium* oocysts and *Giardia* cysts in 3 samples from WTPs A, B, and D. All (oo)cysts observed

Table 2. Results of detection of *Cryptosporidium* spp. and *Giardia* spp. in effluents from wastewater treatment plants (WTP) in eastern Poland

WTP codes	DFA No. of (oo)cysts per litre		Real time PCR		Nested PCR	
	Cryptosporidium	*Giardia*	*Cryptosporidium*	*Giardia*	*Cryptosporidium*	*Giardia*
A	33.0	374.3	(-)	+	(-)	(-)
B	2.6	0.7	+/(-)	(-)	(-)	(-)
C	0.4	1.8	(-)	(-)	(-)	(-)
D	0.0	5.5	(-)	(-)	(-)	+
E	0.0	11.0	(-)	(-)	(-)	(-)
F	0.0	0.0	(-)	(-)	(-)	(-)
G	0.0	660.0	(-)	(-)	(-)	(-)
H	16.5	0.0	(-)	+	(-)	(-)
I	29.4	220.0	(-)	+	(-)	(-)
J	0.0	16.5	(-)	+	(-)	+
K	132.1	6.6	(-)	+	(-)	+
L	154.1	176.2	(-)	+	(-)	+
M	2.2	3.7	(-)	(-)	(-)	(-)
Median	2.2	6.6				
Mean ± S.D.	28.5 ± 52.4	113.6 ± 201.7				

+ = positive, (-) = negative, +/(-) = equivocal (doubtful)

Table 3. Viability assessment of *Cryptosporidium* and *Giardia* isolated from effluent samples of selected WTPs

WTP codes	Viability No. of live/total (oo)cysts (%)	
	Cryptosporidium spp.	*Giardia* spp.
A	1/8 (12.5)	2/4 (50.0)
B	3/5 (60.0)	2/2 (100.0)
C	0/5 (0.0)	0/1 (0.0)
D	2/4 (50.0)	1/2 (50.0)

in the sample from WTP C were recognised as dead (light green or orange/yellow color). The proportions of live/total (oo)cysts determined in each sample are shown in Table 3.

PCR results. Based on detection of β-giardin *Giardia* gene, 4 (30.8%) of the 13 examined samples were positive. PCR based on the fragment 18S SSU rRNA *Cryptosporidium* gene revealed no positive reactions.

In Real Time PCR, positive results for *Giardia* were detected in 5 samples (38.5%) and none in the sample for *Cryptosporidium*, with the exception of one equivocal result for WTP B sample (Tab. 2)

DISCUSSION

The purpose of the presented study was a preliminary assessment of the prevalence of parasitic protozoans *Giardia* and *Cryptosporidium* in finished effluents from selected WTPs located in the Lublin region of eastern Poland. It seems that estimating the efficiency of treatment plants in removing these parasites is important from the aspect of public health [33, 34]. Even well-operated water treatment plants cannot ensure that drinking water will be completely free of *Cryptosporidium* oocysts. Current Polish regulations for treated wastewater and sludge define limits for chemical, bacteriological, and helminth eggs, but do not include regulations for parasitic protozoa [35, 36].

The transmission of *Giardia* and *Cryptosporidium* spp. through contaminated water, food, and mainly the oral-faecal route to humans, is well documented. The cycling of pathogens can occur between humans and also between animals and humans [6, 14]. The potential contamination of water with parasitic protozoa can be initially assessed by the presence of bacteria *Clostridium perfringens* in a sample [37]. However, no statistical correlation was found to exist between the counts of indicator bacteria (*Cl. perfringens*), and occurrence of *Cryptosporidium* or *Giardia*. (Oo)cysts of these parasites are known to be environmentally resistant. *Giardia* cysts remain viable for almost 2 months at 0 – 2 °C [38], and *Cryptosporidium* oocysts for nearly 6 months at 4 °C [39]. (Oo)cysts can be effectively inactivated at the temperature of 55 °C [38, 40]. The number of pathogens can be reduced by various treatments, e.g. aerobic or anaerobic digestion. But after anaerobic digestion, *Cryptosporidium* oocysts have been reported to remain viable at least for 30 days in sludge-treated soil [40].

There is a lack of data concerning the occurrence of *Cryptosporidium* and *Giardia* in treated water effluent from sewage treatment plants in Poland. The presence of protozoan (oo)cysts in effluent samples from the WTPs examined in the presented study suggests that the methods utilized by WTPs are not fully effective. Not completely removed protozoa and the contamination of tap water systems may cause outbreaks of cryptosporidiosis and/or giardiasis. DFA revealed considerable quantities of effluent samples contaminated with *Cryptosporidium* and *Giardia* (61.5% and 84.6%, respectively). The microscopy results of this study revealed that *Giardia* cysts occurred more frequently in the effluents collected from WTPs: A, C, D, E, G, I, J, L and M, whereas *Cryptosporidium* oocysts occurred less frequently, or were absent altogether. The oocysts of *Cryptosporidium* prevailed only in 3 WTPs: B H, and K. Using the USEPA Method 1623, Castro-Hermida and al. [41] also found both parasites in effluent samples from 12 WTPs in Galicia, northwest Spain, and *Giardia* also outnumbered *Cryptosporidium* spp. Similar observations were also made by Dungeni & Momba [42] in South Africa.

The prevalence and concentrations of these parasites found in the presented study are greater compared to some figures reported from Brazil [43], Tunisia [44], Germany [45], Hungary [46], Finland [47], USA [48, 49], Canada [21], and Spain [20]. Tonani et al [43] reported from WTP effluent in Brazil, concentrations of *Cryptosporidium* and *Giardia* ranging from 0 – 1.05 oocysts and 0.45 – 3.5 cysts per litre, respectively. In Tunisia, *Giardia* cysts were detected by the use of IMS-IFA in 4 of 8 treated wastewater samples, whereas *Cryptosporidium* was never detected either by microscopic examination or by IMS-IFA. Nested PCR amplification of the SSU rRNA gene showed that 1 of the 8 treated wastewater samples contained *Cryptosporidium* spp., and 3 of the 8 samples (37.5%) were PCR positive for *G. lamblia* [44]. In Germany, the parasites were detected in about 50% of all treated water samples [45]. Plutzer et al. [46] reported that 42% of treated sewage samples in Hungary contained *Cryptosporidium* oocysts and 67% contained *Giardia* cysts. In the study performed by Rimhanen in Finland [47], only 20.4%

of treated sewage samples contained *Giardia* cysts and 6.8% contained *Cryptosporidium parvum*. Gennacaro et al. [48] found in the USA low numbers of *Cryptosporidium parvum* in WTP effluent amounting to 40% of the examined samples, with an average concentration of 0.07 oocyst/L. McHarry [49], also from the USA, reported a very low concentration of *Cryptosporidium* oocysts in WTP effluent, ranging from 0.011 – 1.19/L. Chauret et al. [21] found in treated sewage in Canada very low levels of *Cryptosporidium* and *Giardia* (0.052 oocyst/L and 3.29 cysts/L, respectively). Rodriguez-Manzano et al. [20] in Spain reported that the concentrations of *Cryptosporidium* and *Giardia* in sewage after the end of the treatment cycle were 0 – 1.6/L and 0 – 13.2/L, respectively.

The presented results are comparable with those obtained by Lim et al. [23] in Malaysia who found in treated sewage 20–80 *Cryptosporidium* oocysts and 1 – 1,462 *Giardia* cysts per litre, and with those reported by Lalancette et al. from Canada, who found in this environment peak values of 89 *Cryptosporidium* oocysts/L and 472 *Giardia* cysts/L [50], with those obtained by McCuin & Clancy in the USA [51] who reported concentrations of *Cryptosporidium* oocysts ranging from below 2 to 86/L, with those found by Bonnadonna et al. [52] in Italy for *Cryptosporidium* ranging from 0 – 82 oocysts/L, and with those obtained Wiandt et al. [19] in France who reported concentrations of *Giardia* cysts ranging from below 1 to 66/L.

Greater numbers of *Giardia* cysts, amounting to 380 – 1,450/L have been reported from Spain by Alonso et al. [53]. Similarly, Robertson et al. [54] noted in WTP effluent in Norway high concentrations of *Cryptosporidium* and *Giardia*, ranging from 4,000 – 36,000 and from 4,000 – 44,000 (extrapolated to per liter), respectively. From effluent samples in Scotland in which oocysts viability was assessed, 46% contained viable oocysts [27].

The method of pellet concentration by centrifugation used in the presented study was simple and inexpensive. IMS and IFA used for detecting *Giardia* and *Cryptosporidium* are implemented in the EPA method 1622/1623 [28]. Limitations of these methods are the selected capacity to detect only *Cryptosporidium* and *Giardia*, and high cost.

The current results did not show full agreement between DFA and PCR. By microscopy, *Cryptosporidium* oocysts were detected in 8, and *Giardia* cysts in 11 samples out of the total of 13 samples. In contrast, by nested PCR, *Cryptosporidium* was detected in none, and *Giardia* in 4 of the 13 samples. In Real Time PCR, 4 positive results were obtained for *Giardia*, whereas for *Cryptosporidium* only 1 doubtful-positive result. These differences could be attributed to the small number of parasites in the samples and/or to the non-full clearance of PCR inhibitors, such as humic and fulvic acids. Generally, from the perspective of molecular epidemiology, the number of *Cryptosporidium* oocysts in treated wastewater was lower than *Giardia* cysts. The differences between DFA and PCR results can result also from some lack of DFA assay specificity, which may have led to an overestimation of the number of (oo)cysts in wastewater by cross-reaction with non-target organisms. This could be due also to the fact that the (oo)cysts could be of a different subspecies, and amplification could not be achieved with the primers used in this study [14, 55, 56]. The expected superiority of PCR method over microscopy was also not confirmed by Guy et al. [4] in relation to *Cryptosporidium* and *Giardia* (oo)cysts. Alonso et al. [53] found by IF microscopy *Cryptosporidium* oocysts in 50% of examined sewage samples, while in RT PCR all samples were negative. Similarly, Mayer & Palmer [10] noted for *Cryptosporidium* from treated sewage less positive results in PCR compared to immunofluorescent microscopy, although for *Giardia,* full agreement was noted between these 2 methods.

The results of the presented study demonstrate that dual-staining with Live/Dead BacLight viability kit accurately stained (oo)cysts of *Cryptosporidium* and *Giardia* as viable or dead. The proportion of viable *Giardia* cysts determined was greater compared to that reported by Wallis et al. [57] which amounted to 17%.

The infective dose for *Cryptosporidium* and *Giardia* is as low as 1 cyst or oocyst, which is quite sufficient to cause infection [14]. In view of our preliminary study, the presence of oocysts and cysts (largely viable) in effluent from WTPs does imply a risk of transmission of protozoan parasites that are of health risk to humans. It may therefore be conclude that additional wastewater purification procedures are necessary.

REFERENCES

1. Fayer R. Cryptosporidium: a water-borne zoonotic parasite. Vet Parasitol. 2004; 126: 37–56.
2. Reynolds KA, Mena KD, Gerba CP. Risk of waterborne illness via drinking water in the United States. Rev Environ Contam Toxicol. 2008; 192: 117–158.
3. Smith HV, Caccio SM, Cook N, Nichols RAB, Tait A. Cryptosporidium and Giardia as foodborne zoonoses. Vet. Parasitol. 2007; 149: 29–40.
4. Guy RA, Payment P, Krull UJ, Horgen PA. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl Environ Microbiol. 2003; 69: 5178–5185.
5. Ryan U, Xiao L, Read C, Zhou L, Lal AA, Pavlasek I. Identification of novel Cryptosporidium genotypes from the Czech Republic. Appl Environ Microbiol. 2003; 69: 4302–4307.
6. Thompson RCA. Giardiasis as a re-emerging infectious disease and its zoonotic potential. Int J Parasitol. 2000; 30: 1259–1267.
7. Thompson RCA, Armson A, Ryan UM. Cryptosporidium: from molecules to disease. Elsevier, Amsterdam 2003.
8. Karanis P, Kourenti C. Waterborne transmission of protozoan parasites: a review of world-wide outbreaks. In: Fourth International Giardia Conference and First Combined Giardia-Cryptosporidium Meeting, 20–24 September 2004, Amsterdam, The Netherlands.
9. Karanis P, Papadopoulou C, Kimua A, Economou E, Kourenti C, Sakkas H. Cryptosporidium and Giardia in natural, drinking and recreational water of northwestern Greece. Acta Hydrochim Hydrobiol. 2002; 30: 49–58.
10. Mayer CL, Palmer CJ. Evaluation of PCR, nested PCR, and fluorescent antibodies for detection of Giardia and Cryptosporidium species in wastewater. Appl Environ Microbiol. 1996; 62: 2081–2085.
11. Nygård K, Schimmer B, Søbstad Ø, Walde A, Tveit I, Langeland N, Hausken T, Aavitsland P. A large community outbreak of waterborne giardiasis-delayed detection in a non-endemic urban area. BMC Public Health. 2006; 6:141.
12. Thompson RCA, Monis PT. Variation in Giardia: implications for taxonomy and epidemiology. Adv Parasitol. 2004; 58: 69–137.
13. Carrington EG, Gray P. The influence of cattle waste and sewage effluent on the levels of Cryptosporidium oocysts in surface waters. Foundation for Water Research, Marlow, Bucks, UK, 1993: 205–208.
14. Caccio SM, De Giacomo M, Aulicino FA, Pozio E. Giardia cysts in wastewater treatment plants in Italy. Appl Environ Microbiol. 2003; 69: 3393–3398.
15. Quintero-Betancourt W, Gennaccaro AL, Scott TM, Rose JB. Assessment of methods for detection of infectious Cryptosporidium oocysts and Giardia cysts in reclaimed effluents. Appl Environ Microbiol. 2003; 69: 5380–5388.
16. Reinoso R, Blanco S, Torres-Villamizar LA, Bécares E. Mechanisms for parasites removal in a waste stabilisation pond. Microb Ecol. 2011; 61: 684–692.
17. Cheng HW, Lucy FE, Graczyk TK, Broaders MA, Tamang L, Connolly M. Fate of *Cryptosporidium parvum* and *Cryptosporidium hominis*

oocysts and *Giardia duodenalis* cysts during secondary wastewater treatments. Parasitol Res. 2009; 105:689–696.
18. Neto RC, Santos LU, Franco RM. Evaluation of activated sludge treatment and the efficiency of the disinfection of Giardia species cysts and Cryptosporidium oocysts by UV at a sludge treatment plant in Campinas, south-east Brazil. Water Sci Technol. 2006; 54: 89–94.
19. Wiandt S, Grimason AM, Baleux B, Bontoux J. Efficiency of wastewater treatment plants at removing Giardia sp. cysts in southern France. Schriftenr Ver Wasser Boden Lufthyg. 2000; 105: 35–42.
20. Rodriguez-Manzano J, Alonso JL, Ferrús MA, Moreno Y, Amorós I, Calgua B, Hundesa A, Guerrero-Latorre L, Carratala A, Rusiñol M, Girones R. Standard and new faecal indicators and pathogens in sewage treatment plants, microbiological parameters for improving the control of reclaimed water. Water Sci Technol. 2012; 66:2517–2523.
21. 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.
22. Gray NF. Biology of wastewater treatment. Imperial College Press, London, UK, 2004.
23. Lim YA, Wan Hafiz WI, Nissapatorn V. Reduction of Cryptosporidium and Giardia by sewage treatment processes. Trop Biomed. 2007; 24: 95–104.
24. Graczyk TK, Lucy FE, Tamang L, Miraflor A. Human enteropathogen load in activated sewage sludge and corresponding sewage sludge end products. Appl Environ Microbiol. 2007; 73: 2013–2015.
25. World Health Organization, Guidelines for Drinking Water Quality; Addendum-Microbial Agents in Drinking Water. 2nd Ed. Geneva 2002.
26. Lisle JT, Rose JB. Cryptosporidum contamination of water in the USA and UK: a minireview. J Water Supply Res Technol. 1995; 44: 103–105.
27. Robertson LJ, Paton CA, Campbell AT, Smith PG, Jackson MH, Gilmour RA, Black SE, Stevenson DA, Smith HV. Giardia cysts and Cryptosporidium oocysts at sewage treatment works in Scotland, UK. Water Res. 2000; 34: 2310–2322.
28. US EPA method 1623, 2001. Cryptosporidium and Giardia in water by filtration/IMS/FA. EPA-821-R-01–025, pp. 1–58.
29. Taghi-Kilani R, Gyürék LL, Millard PJ, Finch GR, Belosevic M. Nucleic acid stains as indicators of Giardia muris viability following cyst inactivation. Int J Parasitol. 1996; 26: 637–646.
30. Caccio SM, De Giacomo M, Pozio E. Sequence analysis of the β-giardin gene and development of a polymerase chain reaction–restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. Int J Parasitol. 2002; 32: 1023–1030.
31. Xiao L, Lal AA, Jiang J. Detection and differentiation of Cryptosporidium oocysts in water by PCR-RFLP. Methods Mol Biol. 2004; 268: 163–176.
32. Santın M, Trout JM, Xiao L, Zho L, Greiner E, Fayer R. Prevalence and age-related variation of *Cryptosporidium* species and genotypes in dairy calves. Vet Parasitol. 2004; 122: 103–117.
33. Lonigro A, Pollice A, Spinelli R, Berrilli F, DiCave D, D'Orazi C, Cavallo P, Brandonisio O. Giardia cysts and Cryptosporidium oocysts in membrane-filtered municipal wastewater used for irrigation. Appl Environ Microbiol. 2006; 72: 7916–7918.
34. Pollice A, Lopez A, Laera G, Rubino G, Lonigro A. Tertiary filtered municipal wastewater as alternative water source in agriculture: a field investigation in southern Italy. Sci Total Environ. 2004; 324: 201–210.
35. Rozporządzenie Ministra Zdrowia z 29 marca 2007 r. w sprawie jakości wody przeznaczonej do spożycia przez ludzi (Dz. U. nr 61, poz. 417).
36. Ustawa z dnia 18 lipca 2001 r. Prawo wodne (Dz. U. z 2005 r. Nr 239, poz. 2019 z późn. zm.).
37. Payment P, Franco E. *Clostridium perfringens* and somatic coliphages as indicators of the efficiency of drinking water treatment for viruses and protozoan cysts. Appl Environ Microbiol. 1993; 59: 2418–2424.
38. deRegnier D, Cole L, Schupp DG, Erlandsen SL. Viability of Giardia cysts suspended in lake, river and tap water. Appl Environ Microbiol. 1989; 55: 1223–1229.
39. Robertson LJ, Campbell AT, Smith HV. Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. Appl Environ Microbiol. 1992; 58: 3494–3500.
40. Whitmore TN, Robertson LJ. The effect of sewage sludge treatment processes on oocysts of *Cryptosporidium parvum*. J Appl Bacteriol. 1995; 78: 34–38.
41. Castro-Hermida JA, Garcıa-Presedo I, Almeida A, Gonzalez-Warleta M, Correia Da Costa JM, Mezo M. Detection of Cryptosporidium spp. and Giardia duodenalis in surface water: A health risk for humans and animals. Water Research. 2009; 43: 4133–4142.
42. Dungeni M, Momba MNB. The abundance of *Cryptosporidium* and *Giardia* spp. in treated effluents produced by four wastewater treatment plants in the Gauteng Province of South Africa. Water SA 2010; 36: 425.
43. Tonani KA, Padula J, Julião FC, Fregonesi BM, Alves RI, Sampaio CF, Beda CF, Hachich E, Segura-Muñoz S. Persistence of Giardia, Cryptosporidium, Rotavirus and Adenovirus in Treated Sewage in São Paulo State, Brazil. J Parasitol. 2013 May 29. [Epub ahead of print]
44. Ben L, Khouja A, Cama V, Xiao L. Parasitic contamination in wastewater and sludge samples in Tunisia using three different detection techniques Parasitol Res. 2010; 107: 109–116.
45. Karanis P, Sotiriadou I, Kartashev V, Kourenti C, Tsvetkova N, Stojanova K. Occurrence of Giardia and Cryptosporidium in water supplies of Russia and Bulgaria. Environ. Res. 2006; 102: 260–271.
46. Plutzer J, Karanis P, Domokos K, Törökné A, Márialigeti K. Detection and characterisation of Giardia and Cryptosporidium in Hungarian raw, surface and sewage water samples by IFT, PCR and sequence analysis of the SSUrRNA and GDH genes. Int J Hyg Environ Health. 2008; 211: 524–533.
47. Rimhanen-Finne R, Ronkainen P, Hänninen ML. Simultaneous detection of Cryptosporidium parvum and Giardia in sewage sludge by IC-PCR. J Appl Microbiol. 2001; 91: 1030–1035.
48. Gennaccaro AL, McLaughlin MR, Quintero-Betancourt W, Huffman DE, Rose JB. Infectious Cryptosporidium parvum oocysts in final reclaimed effluent. Appl Environ Microbiol. 2003; 69: 4983–4984.
49. McHarry MJ. Detection of Giardia in sewage effluent. J Protozool. 1984; 31: 362–364.
50. Lalancette C, Généreux M, Mailly J, Servais P, Côté C, Michaud A, Di Giovanni GD, Prévost M. Total and infectious Cryptosporidium oocyst and total Giardia cyst concentrations from distinct agricultural and urban contamination sources in Eastern Canada. J Water Health. 2012; 10:147–160.
51. McCuin RM, Clancy JL. Occurrence of Cryptosporidium oocysts in US wastewaters. J Water Health. 2006; 4: 437–452.
52. Bonadonna L, Briancesco R, Ottaviani M, Veschetti E. Occurrence of Cryptosporidium oocysts in sewage effluents and correlation with microbial, chemical and physical water variables. Environ Monit Assess. 2002; 75: 241–252.
53. Alonso JL, Amorós I, Cañigral I. Development and evaluation of a real-time PCR assay for quantification of Giardia and Cryptosporidium in sewage samples. Appl Microbiol Biotechnol. 2011; 89:1203–1211.
54. Robertson LJ, Hermansen L, Gjerde BK. Occurrence of Cryptosporidium oocysts and Giardia cysts in sewage in Norway. Appl Environ Microbiol. 2006; 72:5297–5303.
55. Sturbaum GD, Reed C, Hoover PJ, Jost BH, Marshal MM, Sterling CR. Species-Specific, Nested PCR Restriction Fragment Length Polymorphism Detection of Single *Cryptosporidium parvum* oocysts. Appl Environ Microbiol. 2001; 67: 2665–2667.
56. Sulaiman IM., Jiang J, Singh A, Xiao L. Distribution of *Giardia duodenalis* genotypes and subgenotypes in raw urban wastewater in Milwaukee, Wisconsin. Appl Environ Microbiol. 2004; 70: 3776–3780.
57. Wallis PM, Erlandsen SL, Isaac-Renton JL, Olson ME, Robertson WJ, van Keulen H. Prevalence of Giardia cysts and Cryptosporidium oocysts and characterization of Giardia spp. isolated from drinking water in Canada. Appl Environ Microbiol. 1996; 62:2789–2797.