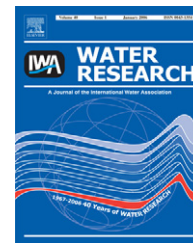


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# Contribution of treated wastewater to the contamination of recreational river areas with *Cryptosporidium* spp. and *Giardia duodenalis*

José Antonio Castro-Hermida<sup>a,\*</sup>, Ignacio García-Preledo<sup>a</sup>, André Almeida<sup>b</sup>,  
Marta González-Warleta<sup>a</sup>, José Manuel Correia Da Costa<sup>b</sup>, Mercedes Mezo<sup>a</sup>

<sup>a</sup>Laboratorio de Parasitología, Centro de Investigaciones Agrarias de Mabegondo-Xunta de Galicia, Carretera AC-542 de Betanzos a Mesón do Vento, Km 7, 5. CP 15318 Abegondo (A Coruña), Spain

<sup>b</sup>Centro de Imunología e Biología Parasitaria-INSA, Porto, Portugal

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## ABSTRACT

Samples of the influent and final effluent from 12 wastewater treatment plants from Galicia (NW, Spain) were analyzed for the presence of *Cryptosporidium* spp. oocysts and *Giardia duodenalis* cysts. All of the plants discharge effluent to a hydrographic basin in which there are numerous recreational areas and fluvial beaches. The samples (25–50 liters) were collected in spring, summer, autumn and winter of 2007. A total of 96 samples were analyzed using techniques included in the US Environmental Protection Agency Method 1623. To identify the genotypes present, the following genes were amplified and sequenced: 18S SSU rRNA (*Cryptosporidium* spp.) and  $\beta$ -giardina (*G. duodenalis*). Both parasites were detected in influent and effluent samples from all treatment plants (100%) throughout the year, and *G. duodenalis* always outnumbered *Cryptosporidium* spp. The mean concentration of *G. duodenalis* per liter of influent was significantly higher ( $P < 0.05$ ) than the mean concentration of *Cryptosporidium* spp. per liter of influent. The mean concentrations of parasites in influent samples ranged from 6 to 350 *Cryptosporidium* spp. oocysts per liter and from 89 to 8305 *G. duodenalis* cysts per liter. In final treated effluent, the mean concentration of parasites ranged from 2 to 390 *Cryptosporidium* spp. oocysts per liter and from 79 to 2469 *G. duodenalis* cysts per liter. The distribution of results per season revealed that in all plants, the highest number of (oo)cysts were detected in spring and summer. *Cryptosporidium parvum*, *Cryptosporidium andersoni*, *Cryptosporidium hominis* and assemblages A-I, A-II, E of *G. duodenalis* were detected. The risk of contamination of water courses by *Cryptosporidium* spp. and *G. duodenalis* is therefore considerable. It is important that wastewater treatment authorities reconsider the relevance of the levels of contamination by both parasites in wastewater, and develop adequate countermeasures.

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## 1. Introduction

*Cryptosporidium* spp. and *Giardia duodenalis* are major causes of diarrheal disease in humans and animals worldwide,

and major causes of protozoan waterborne diseases (Fayer, 2004; Smith et al., 2007; Reynolds et al., 2008). These parasites are transmitted through contaminated water and food, in addition to the classical fecal–oral route. Transmission is

\*Corresponding author. Tel.: +34 981 647 902x248; fax: +34 981 673 656.

E-mail address: [jose.antonio.castro.hermida@xunta.es](mailto:jose.antonio.castro.hermida@xunta.es) (J.A. Castro-Hermida).  
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sustained by both zoonotic and anthroponotic cycles (Fayer et al., 2000; Slifko et al., 2000; Thompson, 2000). The infected hosts, whether humans and animals, shed very large numbers of transmissible stages—oocysts of *Cryptosporidium* spp. and cysts of *G. duodenalis*—in their feces, thereby increasing the degree of environmental contamination. Moreover, the (oo)cysts are insensitive to conventional wastewater treatment processes and disinfectants, at least at the concentrations commonly used in water treatments plants to reduce bacterial contamination (Karanis et al., 2002; WHO, 2002; Center of Disease Control and prevention, 2006), although it has been shown that *G. duodenalis* cysts are less resistant than *Cryptosporidium* spp. oocysts (Sterling, 1990) to higher concentrations of chlorine and ozone. *G. duodenalis* cysts have been shown to survive in water for up to 2 months at temperatures as low as 8 °C (Meyer and Jarroll, 1980) and *Cryptosporidium* spp. oocysts can survive for up to 1 year at 4 °C in artificial seawater (Tamburrini and Pozio, 1999).

Depending on the efficiency of the conventional wastewater treatment at the plants, (oo)cysts may pass through the treatment process and enter surface waters (i.e., sources of drinking water, rivers, in which there may be pumping areas for drinking water treatment plants, or recreational waters), leading to potential infection of humans. Moreover, in industrialized countries, use of treated wastewater for domestic, industrial and agricultural purposes is nowadays the most important method of reusing wastewater, when sanitary and environmental guarantees are given.

*Cryptosporidium* spp. and *G. duodenalis* have been detected in irrigation water, effluents from wastewater treatment plants and biosolids from wastewater treatment plants (Chauret et al., 1999; Cacciò et al., 2003; Quintero-Betancourt et al., 2003), and therefore, there is a risk that reclaimed wastewater applied to agricultural land may contain these as well as other pathogens. Forage food may thus become contaminated, leading to disease in livestock. Infected animals may then amplify the numbers of pathogens and contaminate the environment, including surface waters, or spread food-borne disease if the quality of meat processing and handling is compromised. Infected animals may also serve as reservoirs of the pathogen, and although they may not show obvious signs of disease or clinical symptoms (Castro-Hermida et al., 2005, 2007a, b), they may ultimately spread the pathogens to human food (Hancock et al., 2001) or directly to humans (Enriquez et al., 2001). Furthermore, the infectious dose (ID<sub>50</sub>) has been estimated to be as low as 83–132 oocysts for *Cryptosporidium* spp. (DuPont et al., 1995; Chappell et al., 2006) and 19–50 cysts for *G. duodenalis* (Adam, 2001).

There are no requirements for testing recreational waters for these parasites, although it has been shown that these pathogens can be shed into recreational water during outbreaks (Yoder et al., 2004; Craun et al., 2005; Sunderland et al., 2007). Unlike swimming pools, recreational beach areas have underlying sediment that may contain up to 1000 times as many fecal bacterial or parasitic indicators as the overlying water (Indest, 2003). Disturbance of this sediment may lead to re-suspension of bacterial coliforms and of (oo)cysts, contributing to the higher concentrations in water (Brookes et al., 2004; Fayer, 2004; Giangaspero et al., 2007).

Outbreaks may occur during malfunctioning of treatment plants, which would enable the parasites to bypass the treatment processes more easily. Wastewater treatment plants have the potential to be a source of contamination to watersheds if the effluents are not treated adequately before they are discharged into nearby rivers or ponds. The objectives of the present study were to evaluate the prevalence and concentrations of *Cryptosporidium* spp and *G. duodenalis* in 12 wastewater treatment plants whose effluents are discharged into a hydrographical basin where there are several recreational areas, river beaches and pumping areas for drinking water treatment plants, in order to estimate the efficiency of treatment plants in removing these protozoans and to determine which species and genotype of the parasites are present, by means of a molecular assay.

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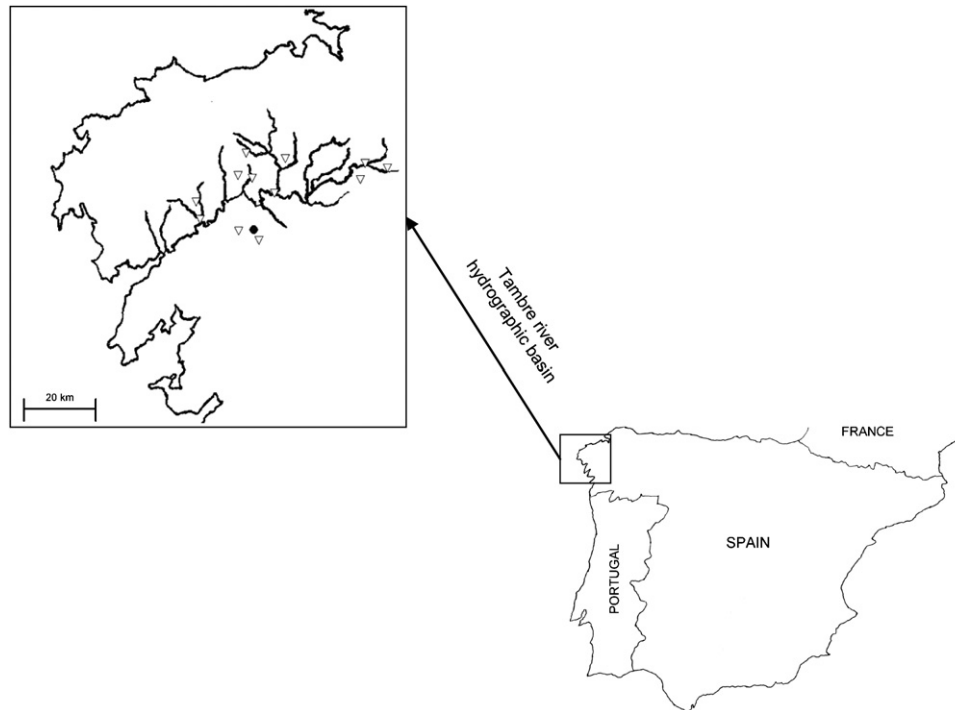
## 2. Material and methods

### 2.1. Sample collection

Samples were collected at 12 wastewater treatment plants (WTP). All of the WTP analyzed are located in the Tambre River hydrographic basin, which is one of the most important basins in this study region (Galicia, NW Spain). The principal river in the basin was declared a Site of Community Importance (SCI), by the European Commission Habitats Directive (92/43/EEC) in 2001. However, all of the final effluents from the WTP are discharged into the hydrographical basin, where there exist several recreational areas, river beaches and pumping areas for drinking water treatment plants, being 185,695 people whose drinking water supply is provided from the principal river (Fig. 1).

Two samples (25–50 liters), one of untreated wastewater (influent) and other of treated water (final effluent), were collected in each WTP during the spring, summer, autumn and winter of 2007 (24 samples per season). To be able to examine the wastewater at the same stage of the treatment process, when collecting the samples, the holding times of each step in the process were respected.

The specific steps in the treatment process used in each of the 12 plants are described in Table 1. The wastewater samples were filtered through Filta-Max filters (IDEXX Laboratories, Inc., Westbrook, ME, USA) with the aid of a motorized pump located on the inlet side of the filter. Samples were taken immediately to the laboratory and processed by the Filta-Max Automatic System (IDEXX Laboratories, Inc., Westbrook, ME, USA). Filtration cartridges were used according to the manufacturer's instructions, and all samples were filtered at recommended flow rates. The filter elution and final concentration of the sample were performed with the same equipment, and by following the manufacturer's instructions. Briefly, a membrane filter (3- $\mu$ m pore size; polysulfone) was placed in the base of the sample concentrator, the filter module was inserted into the apparatus, 600 ml of phosphate-buffered saline (PBS) containing 0.01% Tween 20 (PBST) was added to the reservoir, and the filter housing was unscrewed to allow expansion of the foam pads contained within. The foam pads were washed by pumping the plunger and then transferring the PBST elution volume



**Fig. 1 – Geographic location of the wastewater treatment plants (▽) in the Tambre River hydrographic basin (Galicia; NW Spain). All of them were sampled in this study. Santiago de Compostela (●), with 93,458 inhabitants, is the main city in this area.**

**Table 1 – Main features of the treatment plant processes**

| Plant | Loading/<br>population<br>equivalent | Primary treatment                               | Secondary<br>treatment                             | Tertiary<br>treatment | Use of treated water   |
|-------|--------------------------------------|---|--|-----------------------|--|
| 1     | 30,191                               | Screening, grit separation<br>and sedimentation | Activated sludge and<br>sedimentation              | None                  | Discharged to the river, agriculture<br>irrigation                 |
| 2     | 4652                                 | Screening and grit<br>separation                | Oxidation with O <sub>2</sub><br>and sedimentation | Ultraviolet           | Discharged to the river, agriculture<br>irrigation                 |
| 3     | 2410                                 | Screening                                       | Oxidizing beds                                     | None                  | Discharged to the river, agriculture<br>irrigation                 |
| 4     | 6481                                 | Screening and grit<br>separation                | Activated sludge and<br>sedimentation              | None                  | Discharged to the river, agriculture<br>irrigation                 |
| 5     | 12,294                               | Screening, grit separation<br>and sedimentation | Activated sludge and<br>sedimentation              | None                  | Discharged to the river  |
| 6     | 6526                                 | Screening and grit<br>separation                | Activated sludge and<br>sedimentation              | None                  | Discharged to the river  |
| 7     | 93,458                               | Screening, grit separation<br>and sedimentation | Activated sludge and<br>sedimentation              | Ultraviolet           | Discharged to the river, agriculture<br>irrigation                 |
| 8     | 2289                                 | Screening and<br>sedimentation                  | Biological aerated<br>filters                      | None                  | Discharged to the river, restoration<br>and agriculture irrigation |
| 9     | 4548                                 | Screening and grit<br>separation                | Oxidation with O <sub>2</sub>                      | None                  | Discharged to the river and<br>agriculture irrigation              |
| 10    | 3557                                 | Screening                                       | Oxidation with O <sub>2</sub>                      | None                  | Discharged to the river and<br>agriculture irrigation              |
| 11    | 4580                                 | Screening and grit<br>separation                | Oxidation with O <sub>2</sub><br>and sedimentation | Ultraviolet           | Discharged to the river  |
| 12    | 1496                                 | Screening                                       | Oxidizing beds                                     | None                  | Discharged to the river  |

into the magnetic particle concentrator and filtering the entire volume to approximately 20–25 ml under vacuum. The process was repeated with a second 600-ml volume of PBST,

which was pooled with the first concentrate and then filtered under vacuum to produce a final volume of around 20 ml. The concentrate was then transferred into a 50-ml centrifuge

tube, the filter membrane was transferred to a small sealable plastic bag, and 8–10 ml of PBST added. The membrane was kneaded manually, and the spent wash volume was pooled with the primary eluate. The membrane washing procedure was repeated twice. The sample was resuspended to 50 ml with PBST and centrifuged at 1500g for 5 min. The supernatant was aspirated to 10 ml, and the resuspended pellet was transferred to a Leighton tube and processed by immunomagnetic separation technology (IMS).

## 2.2. Sample analysis for presence of parasites

The IMS procedure was performed as described in US EPA method 1623 (USEPA, 2001). Briefly, each 10-ml sample concentrate was added to a Leighton tube containing 1 ml of  $10 \times$  SL buffer A and 1 ml of  $10 \times$  SL buffer B (Dynabeads<sup>®</sup> GC-Combo, Invitrogen Dynal, A.S., Oslo, Norway). One hundred microliters of *Cryptosporidium* and *Giardia* IMS beads were added to each tube, and samples were incubated for 1 h at room temperature with constant rotation. The Leighton tubes were then placed in a magnetic particle concentrator and gently rocked for 2 min through a  $90^\circ$  angle. The supernatant was decanted, the tubes were removed from the magnetic particle concentrator, and 1 ml of  $1 \times$  SL buffer A was added to each. The tubes were gently rocked to resuspend the bead-(oo)cyst complexes, the suspension was transferred into a 1.5 ml polypropylene tube with a Pasteur pipette, then the tubes were placed in a second magnetic particle concentrator and rocked for 1 min. Supernatants were aspirated, the magnet was removed, and 50  $\mu$ l of 0.1 N HCl was added to each sample, which was vortexed for a minimum of 10 s. Samples were allowed to stand for 10 min in an upright position and vortexed for a further 10 s, and magnets were inserted. The tubes were allowed to stand undisturbed for 2 min. The beads collected at the back of the tube and the acidified suspension was transferred to the wells, of a two-welled slide, containing 5  $\mu$ l 1.0 N NaOH.

## 2.3. Staining procedure for recovered (oo)cysts

The samples were dried, fixed in acetone, and 50  $\mu$ l of fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* sp. and anti-*Giardia* sp. MAbs (Aqua-Glo; Waterborne Inc., New Orleans, LA) was placed in each well. The slides were placed in a humidified chamber and incubated (37 °C for 45 min), then excess FITC-MAB was aspirated off. Any remaining FITC-MAB was removed by adding 50  $\mu$ l of PBS to each well, allowing the slides to stand for 5 min, and aspirating the excess PBS. A 50  $\mu$ l aliquot of 4',6'-diamidino-2-phenylindole (DAPI) solution (0.4  $\mu$ g/ml in PBS) was placed in each well. The slides were allowed to stand at room temperature for 15 min, and excess DAPI solution was removed by washing the slides twice in PBS and once in distilled water. The slides were placed in the dark until dry, a 10  $\mu$ l aliquot of mounting medium (2% diazabicyclooctane [DABCO] in 60% glycerol 40% PBS) was placed in each well, and slides were sealed for subsequent examination under epifluorescence optics, as described in method 1623 (USEPA, 2001). *Cryptosporidium* sp. oocysts were identified on the basis of their size, shape, and the presence of a suture on the oocyst

wall, at a magnification of  $\times 400$  using a Nikon Eclipse 90i microscope (Nikon Corporation, Tokyo, Japan). *Giardia* sp. cysts were identified by their size, shape and the pattern and intensity of immunofluorescence staining (i.e., bright green fluorescence of the cyst wall). Parasites in each slide well were enumerated, and the mean number obtained for the replicates was used to extrapolate to concentrations of parasites per liter of sample. Positive and negative controls, as recommended in the Method 1623, were used.

According to McCuin and Clancy (2003), using Filta-Max system and IMS, the mean recovery percentages of *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts from source water samples, were  $50 \pm 13\%$  and  $41 \pm 9\%$ , respectively.

## 2.4. DNA extraction

For selected samples that contained relatively high concentrations of nucleated (oo)cysts, the remaining untreated wastewater (influent) or final effluent (which had not been examined) was concentrated by centrifugation. The parasites were isolated by IMS (Dynabeads<sup>®</sup> GC-Combo, Invitrogen Dynal, A.S., Oslo, Norway), according to the manufacturer's instructions; the beads were also washed before dissociation of the beads and parasites, and the isolated parasites were resuspended in Tris-EDTA buffer. DNA was isolated with a QIAamp<sup>®</sup> DNA Mini kit (QIAGEN GmbH, Germany), according to the manufacturer's instructions.

## 2.5. Gene amplification and sequencing

*Cryptosporidium* species and genotypes were determined by a technique based on PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the small-subunit (SSU) ribosomal RNA gene (18S SSU rRNA). A two-step nested PCR protocol was used to amplify an 830 bp fragment of the 18S SSU rRNA gene with primers 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCTAATCCTTCGAAACAG GA-3' for primary PCR and 5'-GGAAGGGTTGTATTATTAGATAAAG-3' and 5'-AAGGAGTAA GGAACAACCTCC A-3' for secondary PCR (Xiao et al., 1999). The primary PCR mixture contained  $1 \times$  PCR buffer, 6 mM  $MgCl_2$ , 200  $\mu$ M of (each) deoxynucleoside triphosphate, 100 nM (each) primer, 2U of Taq polymerase (New England BioLabs), and 10  $\mu$ l of purified DNA in a final volume of 50  $\mu$ l. Each of 45 cycles consisted of 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 1 min 30 s after an initial hot start at 94 °C for 4 min and ending with 72 °C for 7 min. A secondary PCR product was then amplified from 5  $\mu$ l of the primary PCR. The secondary PCR mixture was identical except that the concentration of  $MgCl_2$  was 3 mM. Each of 45 cycles consisted of 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 1 min after an initial hot start at 94 °C for 4 min and ending with 72 °C for 7 min.

For molecular typing of *G. duodenalis* a semi-nested PCR and partial sequencing of the  $\beta$ -giardin locus were performed (Cacciò et al., 2002). In the primary reaction, a 753 bp fragment was amplified with the forward primer G7 5'-AAGCCCGAC-GACCTCACCCGCGAGTGC-3', 3' and the reverse primer G759 5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3'. In the sequential semi-nested PCR reaction, a 384 bp fragment was amplified with the forward inner primer G376 5'-CATAACGACGC-CATCGCGCTCTCAGGAA-3' and the reverse primer G759, as

previously described by Cacciò et al. (2002). The PCR mix contained 1 × PCR buffer, 3 mM MgCl<sub>2</sub>, 200 μM (each) deoxynucleoside triphosphate, 200 nM of each primer, 2.5 units of Taq polymerase (New England BioLabs), and 5 μl of purified DNA as a template for primary steps and 2.5 μl of primary PCR product for secondary steps, in a final volume of 50 μl. PCR was performed as follows: initial denaturation step of 94 °C for 5 min, 40 cycles of 30 s at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s and final extension cycle of 7 min at 72 °C.

In all cases, for each reaction, a negative control (water) and a positive control were added. The PCR products were resolved in to 1.2% agarose gel electrophoresis, stained with ethidium bromide and the gel image recorded under UV light. Gel images were captured by a gel documentation system (Gel Doc XR system, BioRad).

Following successful PCR, the products were purified (GFX™ PCR DNA and Gel Band Purification Kit, GE Healthcare Europe GmbH, Germany) and sequenced on both strands (MWG Biotech, Germany). Chromatograms and sequences were examined with Chromas (<http://www.techneleysium.com.au/chromas.html>) and BioEdit (<http://www.mbio.ncsu.edu/BioEdit/page2.html>). Sequence searches were conducted with BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

## 2.6. Statistics

The Kolmogorov–Smirnov test was used to check the normality of the data. For normally distributed data differences in the concentrations of *Cryptosporidium* spp. and *G. duodenalis* in the final effluents from the influents in the WTPs throughout the year were compared by pairwise multiple comparison procedures (Student–Newman–Keuls method) and one-way ANOVA. Nevertheless, for results that did not fulfill assumptions of normality, the Kruskal–Wallis test (nonparametric ANOVA) and Dunn's multiple comparison test were applied.

The tests were carried out with GraphPad InStat® for Windows, version 3.05, and GraphPad Software, San Diego, CA, USA. Differences in the variables were considered significant at the 0.05 level of confidence.

## 3. Results

### 3.1. Prevalence of protozoans in wastewater samples

A total of 96 samples were collected from the untreated wastewater (influent) and final effluent of WTPs during a period of 1 year. *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts were detected in both influent and effluent samples from all plants (100%) throughout the year, and *G. duodenalis* always outnumbered *Cryptosporidium* spp.; moreover, the mean concentration per liter of *G. duodenalis* was significantly higher ( $P < 0.05$ ) than the mean concentration per liter of *Cryptosporidium* spp. in the influent. The mean concentrations of *Cryptosporidium* spp. oocysts per liter of influent sample ranged from 6 to 350 and of *G. duodenalis* cysts per liter, from 89 to 8305. In final treated effluent, the mean concentration of *Cryptosporidium* spp. oocysts per liter of sample ranged from 2 to 390 and of *G. duodenalis* cysts per liter, from 79 to 2469. The concentration of (oo)cysts per liter of untreated wastewater (influent) throughout the year did not differ significantly among the WTPs (Table 2).

In addition, the concentration of (oo)cysts per liter of final effluent throughout the four seasons of the year did not differ significantly among the WTPs, and therefore the treatments at each WTP were ineffective at removing these enteropathogens (Tables 1 and 2). Surprisingly, in four of the WTPs (33%) it was found that the concentration of (oo)cysts per liter was higher in the treated than in untreated water. Thus, in plants 1, 3 and 12, the concentration of *Cryptosporidium* spp. oocysts in the influent and effluent samples were, respectively 350

**Table 2 – Geometric mean, maximum (max) and minimum (min) numbers of *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts in the influent and effluent samples of wastewater treatment plants over a period of 12 months**

| Plant no. | Oocysts per liter |     |     |                  |      |     | Cysts per liter  |        |      |                  |      |     |
|-----------|-------------------|-----|-----|------------------|------|-----|------------------|--------|------|------------------|------|-----|
|           | Influent (n = 4)  |     |     | Effluent (n = 4) |      |     | Influent (n = 4) |        |      | Effluent (n = 4) |      |     |
|           | Mean              | Max | Min | Mean             | Max  | Min | Mean             | Max    | Min  | Mean             | Max  | Min |
| 1         | 350 <sup>a</sup>  | 970 | 16  | 390              | 1120 | 20  | 6462             | 10,000 | 3450 | 1245             | 3600 | 227 |
| 2         | 138               | 400 | 3   | 85               | 200  | 20  | 2533             | 4000   | 1440 | 2469             | 6000 | 200 |
| 3         | 7 <sup>a</sup>    | 20  | 1   | 105              | 260  | 2   | 262 <sup>a</sup> | 587    | 39   | 803              | 1184 | 13  |
| 4         | 12                | 23  | 3   | 3                | 5    | 2   | 927              | 2667   | 3    | 79               | 244  | 2   |
| 5         | 21                | 49  | 2   | 17               | 50   | 1   | 4240             | 8000   | 240  | 553              | 1100 | 195 |
| 6         | 62                | 178 | 4   | 33               | 94   | 2   | 3175             | 3920   | 2400 | 1136             | 2000 | 384 |
| 7         | 72                | 248 | 1   | 12               | 35   | 2   | 8305             | 14,400 | 2240 | 758              | 1867 | 34  |
| 8         | 15                | 46  | 1   | 6                | 16   | 1   | 89 <sup>a</sup>  | 164    | 4    | 238              | 578  | 24  |
| 9         | 6                 | 8   | 4   | 2                | 3    | 1   | 3465             | 9000   | 800  | 341              | 880  | 3   |
| 10        | 56                | 160 | 1   | 7                | 20   | 1   | 3124             | 10,000 | 85   | 707              | 1440 | 50  |
| 11        | 128               | 380 | 1   | 18               | 54   | 1   | 2480             | 4000   | 320  | 723              | 973  | 400 |
| 12        | 31 <sup>a</sup>   | 112 | 1   | 47               | 120  | 1   | 129 <sup>a</sup> | 448    | 2    | 1204             | 4360 | 5   |

<sup>a</sup> The concentration of (oo)cysts per liter was higher in the final treated effluent than in the untreated wastewater (influent); n: number of water samples.

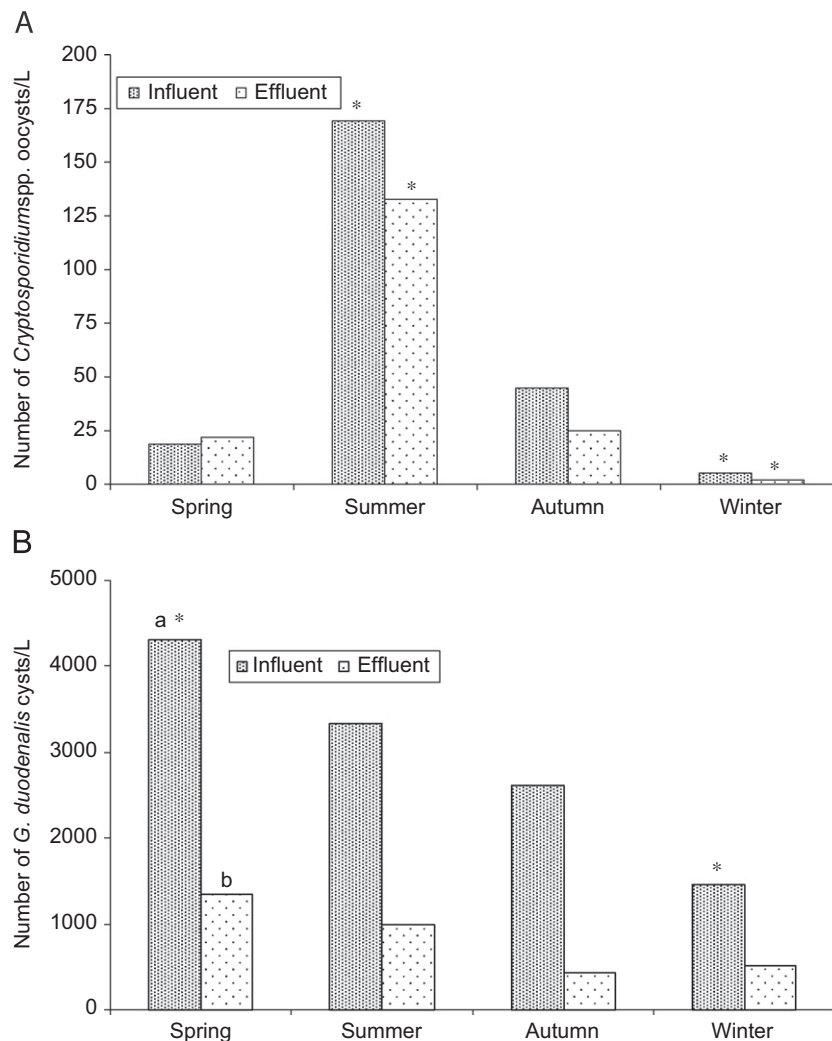
and 390; 7 and 105, and 31 and 47 oocysts per liter, whereas in plants 3, 8 and 12, the concentrations of *G. duodenalis* cysts were, respectively, 262 and 803; 89 and 238, and 129 and 1204 cysts per liter (Table 2). In plants 3 and 12 the primary treatment did not include grit separation and sedimentation, and the secondary treatment consisted of filter beds or oxidizing beds and no disinfection process was applied. This type of sewage treatment was the least efficient at removing the pathogens in comparison with those used in the other plants, although the differences were not statistically significant.

The distribution of results by season revealed that in all WTPs, the highest numbers of (oo)cysts were detected in spring and summer (Fig. 2A and B). It was observed that there was a significantly higher ( $P < 0.05$ ) incidence of positive *Cryptosporidium* spp. oocyst samples in summer than in winter both for the influent and effluent, and there were no significant differences for the other two seasons (Fig. 2A). In the case of *G. duodenalis* the concentrations of cysts per liter was significantly higher ( $P < 0.05$ ) in the

influent collected in spring than in that collected in winter (Fig. 2B).

All of the treatment processes carried out in the 12 WTPs during the different seasons were inefficient as there were no significant differences in the concentrations of (oo)cysts in the effluent and influent samples. A significant reduction ( $P < 0.05$ ) in the number of *G. duodenalis* cysts in the final effluent (1398 cysts per liter) relative to the untreated wastewater (4600 cysts per liter) was only observed in the spring. However, despite this significant difference, the large numbers of cysts that are still being discharged to the river and used for agriculture irrigation during this time of year indicate that the treatments are ineffective.

Comparison of the two seasons in which the highest numbers of parasite forms were detected, revealed that in spring the concentration of both forms in four of the WTP (30%) was higher in the treated water (16–120 oocysts per liter; 326–6000 cysts per liter) than in the untreated water (1–20 oocysts per liter; 2–1440 cysts per liter), although the differences were not significant. At this time of year, the

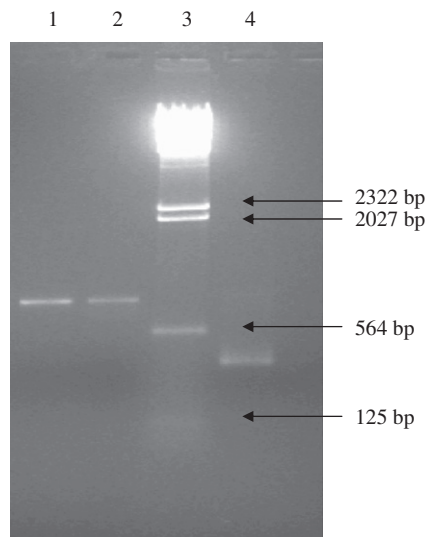


**Fig. 2 – Removal efficacy of *Cryptosporidium* spp. oocysts (A) and *G. duodenalis* cysts (B) by wastewater treatment plants, according to season. Means in the same season with a different letter are significantly differences. Means in different season with an asterisk are significantly different between each other.**

potential contamination of the hydrographic basin (considering only the final effluent) was between 1 and 120 *Cryptosporidium* spp. oocysts per liter (mean 22) and between 18 and 6000 *G. duodenalis* cysts per liter (mean 1.342) (Fig. 2A and B). In summer, at four WTPs (30%) the concentration of oocysts was slightly higher in the treated effluent (20–1120 oocysts per liter) than in the untreated water (3–970 oocysts per liter). A similar situation was observed at three WTPs (23%) for *G. duodenalis* (436–11,840 cysts per liter compared with 64–587 cysts per liter). Contamination of the hydrographic basin by *Cryptosporidium* spp. was significantly higher ( $P < 0.05$ ) in summer, and concentrations of between 1 and 1120 oocysts per liter (mean 133) were detected. At this time of year there was also an increase in the number of *G. duodenalis* cysts, which ranged between 140 and 11,840 cysts per liter (mean 1420). However, this increase was not statistically significant.

### 3.2. Molecular identification of parasites

PCR amplification of 18S SSU rRNA gene sequences was successful for samples containing *Cryptosporidium* spp.



**Fig. 3 – Typical agarose gel with the diagnostic profile for the 18S SSU rRNA gene from *Cryptosporidium* spp. (lanes 1–2) and the b-giardin gene from *G. duodenalis* (lane 4). DNA ladder from Lambda DNA-HindIII digest (lane 3).**

oocysts. For *G. duodenalis* isolates, PCR amplification of the 753 bp fragment of the  $\beta$ -giardin gene was performed (Fig. 3). Samples that contained relatively high concentrations of nucleated (oo)cysts were selected. These samples were collected from the untreated wastewater (influent) and final effluent of the 12 WTPs during the spring and summer. At least two PCR products from each plant were sequenced.

As shown in Table 3, *C. hominis* was detected in the influent samples from 5 (41%) WTPs, and corresponded to the highest loading/population equivalent (Table 1); *C. parvum* and *C. andersoni* in four (33%) and three (25%) of the plants, respectively. For *G. duodenalis*, cysts of assemblage A-I and A-II were detected in two (16%) and three (25%) of the plants, respectively. Nevertheless, in another five samples (41%) two assemblages (A-I+E) were detected and in another two samples (16%), assemblages A-II+E were detected.

The results obtained after sequencing and/or by PCR-restriction fragment length polymorphism of the samples of (oo)cysts in the final effluent were similar to those identified in the influent. Nevertheless, in two WTPs (16%) *C. hominis* was identified in the final effluent and *C. parvum* in the influent; in another two WTPs (16%) *C. parvum* was the predominant species in the final effluent and *C. andersoni* was detected in the untreated wastewater (Table 3).

## 4. Discussion

*Cryptosporidium* spp. and *G. duodenalis* enter the environment in feces from land-dwellers (both human and non-human hosts). Proper management, treatment and dispersal of human feces and animal manures are essential to maintain clean and safe drinking and recreational water. The enormity of the problem of keeping water clean and safe may seem overwhelming when the volume of feces that enters the environment is considered. The encysted forms of these pathogenic protozoa can be carried via wastewater run-off and rainfall-initiated run-off from urban, suburban and rural landscapes, into untreated waters used for agriculture, recreation and, in some locations, for drinking. Moreover, (oo)cysts can withstand normal water disinfection processes, and they have been found in significant quantities in the final effluents discharged from wastewater treatment plants (Cacciò et al., 2003; Fayer, 2004; Montemayor et al., 2005; Robertson et al., 2006; Rueker et al., 2007).

**Table 3 – Identification of species and genotypes of *Cryptosporidium* and *G. duodenalis* detected in the influent and effluent samples from wastewater treatment plants**

| Plant no. | Influent                    |                      | Effluent                    |                      |
|-----------|-----------------------------|----------------------|-----------------------------|----------------------|
|           | <i>Cryptosporidium</i> spp. | <i>G. duodenalis</i> | <i>Cryptosporidium</i> spp. | <i>G. duodenalis</i> |
| 1,7       | <i>C. hominis</i>           | A-II                 | <i>C. hominis</i>           | A-II                 |
| 2, 12     | <i>C. parvum</i>            | A-I+E                | <i>C. hominis</i>           | A-I+E                |
| 4,5,6     | <i>C. hominis</i>           | A-I+E                | <i>C. hominis</i>           | A-I+E                |
| 9         | <i>C. andersoni</i>         | A-II                 | <i>C. andersoni</i>         | A-II                 |
| 3,8       | <i>C. andersoni</i>         | A-II+E               | <i>C. parvum</i>            | A-II+E               |
| 10,11     | <i>C. parvum</i>            | A-I                  | <i>C. parvum</i>            | A-I                  |

This study examined the prevalence and concentrations of *Cryptosporidium* spp. and *G. duodenalis* in 12 wastewater treatment plants that discharge their effluents into a hydrographic basin that includes several recreational areas, river beaches and areas for pumping water to drinking water treatment plants. The aim of the study was to estimate the efficiency of treatment plants in removing these protozoans and to determine the species and genotype of these parasites, by means of a molecular assay, in an attempt to identify host sources of *Cryptosporidium* spp. and *G. duodenalis* that contribute to contamination events and to evaluate their public health significance. Analysis of samples from 12 plants revealed that (oo)cysts were present in all influents and final effluent from WTPs (100%) throughout the year. The occurrence in untreated wastewater and treated final effluents samples are higher than reported in other countries (Bukhari et al., 1997; Cacciò et al., 2003; Robertson et al., 2006; Lim et al., 2007). The results for untreated wastewater demonstrate that *Cryptosporidium* spp. and *G. duodenalis* infections are widespread throughout Galicia, with giardiasis being more widespread and occurring at a higher intensity of infection than cryptosporidiosis. This is consistent with epidemiological data from other countries (Giovanni et al., 2006; Robertson et al., 2006; Lim et al., 2007).

The numbers of parasites in the influent of a WTP depend on the size of the community served, as well as the rate of infection within the population (Smith and Rose, 1990). The greater the size of population served, the higher the probability that the population will include an infected individual (Robertson et al., 2006; Lim et al., 2007). Nevertheless, in this study there was no direct relation between the population loading equivalent and the parasite concentrations in the WTPs. This may be due to the location of some of the WTPs as the hydrographic basin includes farming areas, especially cattle farms, and numerous pastures and crops to which fertilizers are applied during the year, as well as hunting areas, for both large and small game, and there may therefore be uncontrolled contamination via animals (especially wild animals), which are important sources of parasites in terms of wastewater contamination (Rueker et al., 2007). Wild and farmed animals were frequently observed during the study, residing in the natural vegetation buffer zones or directly within agricultural and urban settings. Moreover, in some small towns and villages (< 5000 inhabitants) industrial wastewater, often including animal excrements, are treated in the same WTPs where human sewage is treated, and obviously this practice favors the transmission of the zoonoses.

The determination of the species and genotype of these parasites by means of a molecular assay helps in the identification of host sources of *Cryptosporidium* spp. and *G. duodenalis* that contribute to contamination. Nevertheless, caution is required when extrapolating molecular data from water samples to determine host sources of contamination. In studies that determine the host specificity of *Cryptosporidium* spp. and *G. duodenalis*, a direct association between the host being studied and the fecal material used for typing parasites has been observed (Perz and LeBlancq, 2001; Ryan et al., 2003; Zhou et al., 2004; Castro-Hermida et al., 2006a, b). This direct association does not exist for isolates from water

samples. Therefore, the host source can only be inferred from available host–parasite information and molecular phylogenetics, which can then be further supported by land use information. For example, the host source of *C. parvum* cannot be inferred from detection of a single occurrence; however, when the known host sources of *C. parvum* are considered along with land use analysis, the data suggest that cattle, deer and humans are the most probable sources within the watershed under study (Xiao et al., 2004).

*Cryptosporidium hominis* (formerly *C. parvum* genotype I or human, involved in the anthroponotic route of transmission) was detected in the influent from plants 1 and 7, as well as assemblage A of *G. duodenalis* (genotype A-II), responsible for anthroponotic transmission of infection, suggesting that the majority of (oo)cysts in the untreated wastewater originated from human infections. Although there is no available data on the presence of cases of human cryptosporidiosis and giardiasis in the area of influence of these two WTPs, extrapolation from the analytical data, combined with WTP technical data, enable estimation of the numbers of infected individuals, assuming that (oo)cysts in the influent originated from relatively heavy, and therefore symptomatic, infections. Obviously, (oo)cysts in untreated wastewater will not only be derived from symptomatic persons but also from asymptomatic individuals excreting lower number of (oo)cysts; this is probably particularly so for *G. duodenalis*, which is recognized as a cause of chronic infections. In the other 10 WTPs, the existence of a single definite source of infection cannot be identified. Thus in plants 4, 5 and 6, *C. hominis* (anthroponotic route of transmission) was detected, but not assemblage A of *G. duodenalis* (genotype A-I), which infects humans, domestic ruminants, dogs, cats, beavers, rats and other animals or assemblage E *G. duodenalis* responsible for infections in domestic ruminants, cats and rats. In such cases, in which species or genotypes responsible for anthroponotic and zoonotic routes of transmission are detected it is possible that there is a higher predominance of species of animal or of human origin in specific areas. In plants 9, 3 and 8 where *C. andersoni* (which infects the abomasum of juvenile and mature cattle and has been identified as a cause of reduced milk production but is not known to affect animals other than cattle (Anderson, 1998)) was detected along with assemblage A of *G. duodenalis* (genotype A-II) (responsible for anthroponotic transmission) and assemblage E of *G. duodenalis* (plants 3 and 8), it is possible that there is a higher proportion of bovine than human cryptosporidiosis in the area of influence of the plant, and a higher proportion of human giardiasis than animal giardiasis. This information was obtained by examination of the influent, which provides a more representative indication of endemic infection than epidemiological data, as it takes into account all of the asymptomatic carriers and misdiagnosed cases that usually escape detection. Nevertheless, and as previously mentioned, in some cases it is not possible to detect the host source of contamination specifically. For example, in plants 2, 10, 11 and 12 we detected *C. parvum* and assemblage A of *G. duodenalis* (genotype A-I) responsible for zoonotic transmissions and assemblage E of *G. duodenalis* (plants 2 and 12), which principally infect domestic ruminants. It may therefore be speculated that the random distribution of (oo)cysts may



result in one isolate predominating on one occasion and another isolate predominating on another. These results suggest that isolate heterogeneity within a sample should not be excluded unless the PCR analysis is repeated, either with the same or different primer sets.

The presence of high mean concentrations of *Cryptosporidium* spp. oocysts (2–390 oocysts per liter) and *G. duodenalis* cysts (79–2469 cysts per liter), most of them DAPI+ (87%), in the final effluent is of concern to public health because the effluent is discharged directly into the river and in some cases the treated water is used for land restoration and agriculture irrigation, thereby leading to environmental contamination with these pathogenic protozoans. This poses health risks when the river water is used for household consumption and use in communities downstream from the point of discharge. Furthermore, the hydrographic basin not only includes recreational areas, river beaches and pumping areas for drinking water treatment plants, but the main river also flows into an estuary where bivalve molluscs are cultured for human consumption. A high prevalence of *Cryptosporidium* spp. and *G. duodenalis* in bivalve molluscs from this shellfish-farming area has been reported (Gómez-Couso et al., 2005, 2006). As regards the determination of species and genotypes of these parasites by means of a molecular assay in the effluent, the species detected were similar to those detected in the influent, except in plants 2 and 12, in which *C. parvum* was detected in the untreated wastewater and *C. hominis* in the final effluent. This may be attributed to settlement of (oo)cysts, an effect previously shown to reduce rates of incidence (Brookes et al., 2005). The data reinforce the complexity associated with contamination events within a plant and the challenges associated with capturing and accounting for these dynamic spatial and temporal variations by grab sampling methods. Another possible factor is the transportation of (oo)cysts in overland flow within a WTP. The sediment and (oo)cysts are subjected to deposition when present in overland flow or low flow, as in the tanks involved in primary and secondary treatment processes. The low settling velocities indicate that (oo)cysts will settle out of suspension very slowly. In cases where (oo)cysts are attached to particles, settling of (oo)cysts will be linked to the settling velocity of the particles (Searcy et al., 2005; Dai and Boll, 2006). It is thus possible that once the aeration systems in the secondary treatment begin to operate, the (oo)cysts will be resuspended, thereby favoring their exit in the effluent, and therefore the presence of one species or another may depend on the higher concentration of a species or genotype, or may simply be a random process. Moreover, these reasons could explain that in some WTPs there was more pathogens leaving the station after the treatment than coming to that station.

Although *Cryptosporidium* spp. and *G. duodenalis* were found in all of the wastewater samples from the 12 treatment plants throughout the year, the highest numbers of (oo)cysts were found in the spring and summer. Although similar seasonal patterns have been reported by some authors (Isaac-Renton et al., 1996; Cacciò et al., 2003; Montemayor et al., 2005; Carmena et al., 2007), they have not been confirmed by others (Hashimoto et al., 2001; Robertson and Gjerde, 2001) and thus it is not clear whether or not seasonality is a general feature of *Cryptosporidium* spp. and *G. duodenalis* contamina-

tion. In the present study, the increase in parasitic forms detected in spring and summer may be due to agricultural activities (fertilization of pastures with animal manure, lambing, cattle farming, calving, and extensive pasturing of cattle, especially adult cows and cattle). At these times of year many people visit river beaches and recreational areas and take part in aquatic sports and fishing. There are touristic areas all along the hydrographic basin, and the WTPs that serve these areas are not large enough to treat the amount of sewage generated, especially in summer, and therefore the treatment of the wastewater is not adequate.

The type of treatment process plays an important role in determining whether the numbers of *Cryptosporidium* spp. and *G. duodenalis* parasites are reduced. In this study, we showed that the average removal efficiencies at these 12 wastewater treatment plants, which have both primary and secondary treatment processes, ranged between 16% and 86% for *Cryptosporidium* spp. and between 2% and 90% for *G. duodenalis*. These rates of efficiency are consistent with those reported for other treatment plants that use similar processes (Cacciò et al., 2003; Montemayor et al., 2005; Robertson et al., 2006; Lim et al., 2007). Nevertheless, such estimates should be treated with caution. In this study, there were no significant differences in the concentrations of (oo)cysts per liter of effluent or influent at the different times of year. Moreover, as the estimated parasite loads at these WTPs were considerable, particularly for *G. duodenalis*, parasite discharge in the effluent may also be considerable, and thereby represent a risk to human and animal health.

Different studies involving WTPs where only primary treatments are carried out or where each treatment was examined individually revealed generally low removal efficiencies in the primary steps (Casson et al., 1990; Robertson et al., 2000). Primary treatment involves removal of materials, including fats, oils, sand, gravel and rocks, which are easily collected and disposed of. This step is done entirely with machinery. In this study, plants 1, 5, 7 and 8 apply a sedimentation stage in which the sewage is allowed to pass slowly through large tanks, known as “primary clarifiers” or “primary sedimentation tanks”. The tanks are large enough for fecal solids to settle, and for material such as grease and oils to rise to the surface and be skimmed off. The main purpose of the primary stage is to produce a generally homogeneous liquid that can then be treated biologically, and a sludge that can be treated or processed separately. In Galicia, some WTPs use primary treatment processes only, and it should be assumed that in such WTP, removal of parasites from the sewage influent is minimal because this is not the aim of the primary treatment. Most research dealing with parasite removal in WTPs have investigated plants with different secondary or tertiary treatments and have reported removal efficiencies of around 90% for *G. duodenalis* cysts (Cacciò et al., 2003; Robertson et al., 2006; Lim et al., 2007), with removal of *Cryptosporidium* spp. usually lower and more variable. In the present study, the percentage removal efficiencies were highest in plant 7 and ranged between 83% and 90% for *Cryptosporidium* spp. and *G. duodenalis*, respectively; perhaps the use of activated sludge, sedimentation and ultraviolet disinfection resulted in a greater reduction in numbers of (oo)cysts than achieved with other treatments

(oxidation with O<sub>2</sub>, oxidizing beds or biological filters). Nevertheless, in a study of the rate of removal of *C. parvum* oocysts from sewage subjected to laboratory simulation of activated sludge treatment (80–84%), it was demonstrated that infection developed in mice inoculated with the treated sewage, indicating that the remaining oocysts maintained their infectivity and were present in sufficient numbers for infection to occur (Villacorta-Martínez de Maturana et al., 1992). However, in a recent study Graczyk et al. (2007) indicated that sludge activation process is an effective treatment that significantly decreases the number of infectious human intestinal protozoan pathogens. Further research is therefore required to determine the most effective treatment for reducing the concentrations of (oo)cysts in the effluent. The present results may be biased by several factors, including the underestimation of (oo)cyst concentrations due to the efficiency of the techniques, the limited number of samples examined, and the different volume of water treated in each plant. For some WTPs (1, 3, 8 and 12), the concentration of (oo)cysts per liter of effluent was greater than the concentration per liter of influent collected on the same day, which may be due to some alteration in the treatment processes.

## 5. Conclusions

The present study therefore demonstrates the importance of the parasitological control of effluents from WTPs and the need for regulations to establish acceptable concentrations of (oo)cysts based on the posterior use of wastewaters, i.e., whether recycled for agricultural purposes or for industry. Moreover, it is important that wastewater treatment authorities rethink the relevance of levels of *Cryptosporidium* spp. and *G. duodenalis* contamination in wastewater, and that they develop appropriate countermeasures.

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