Seeking the reuse of effluents and sludge from conventional wastewater treatment plants: analysis of the presence of intestinal protozoa and nematode eggs.

María Benito[a,b]; Carmen Menacho[a,b,c]; Patricia Chueca[b]; María P. Ormad[a,c]; Pilar Goñi[b,c]*

[a]*Department of Chemical Engineering and Environmental Technologies, EINA, University of Zaragoza, C/María Luna 3, 50018 Zaragoza, Spain*
[b]*Area of Parasitology, Department of Microbiology, Preventive Medicine and Public Health, Faculty of Medicine, University of Zaragoza, C/Domingo Miral s/n, 50009 Zaragoza, Spain*
[c]*Water and Environmental Health Research Group, Environmental Sciences Institute (IUCA), University of Zaragoza, Zaragoza, Spain*

*Corresponding author: Pilar Goñi. *Area of Parasitology, Department of Microbiology, Preventive Medicine and Public Health, Faculty of Medicine, University of Zaragoza, C/Domingo Miral s/n, 50009 Zaragoza, Spain.* E-mail:pgoni@unizar.es. Telephone number: +34976761692

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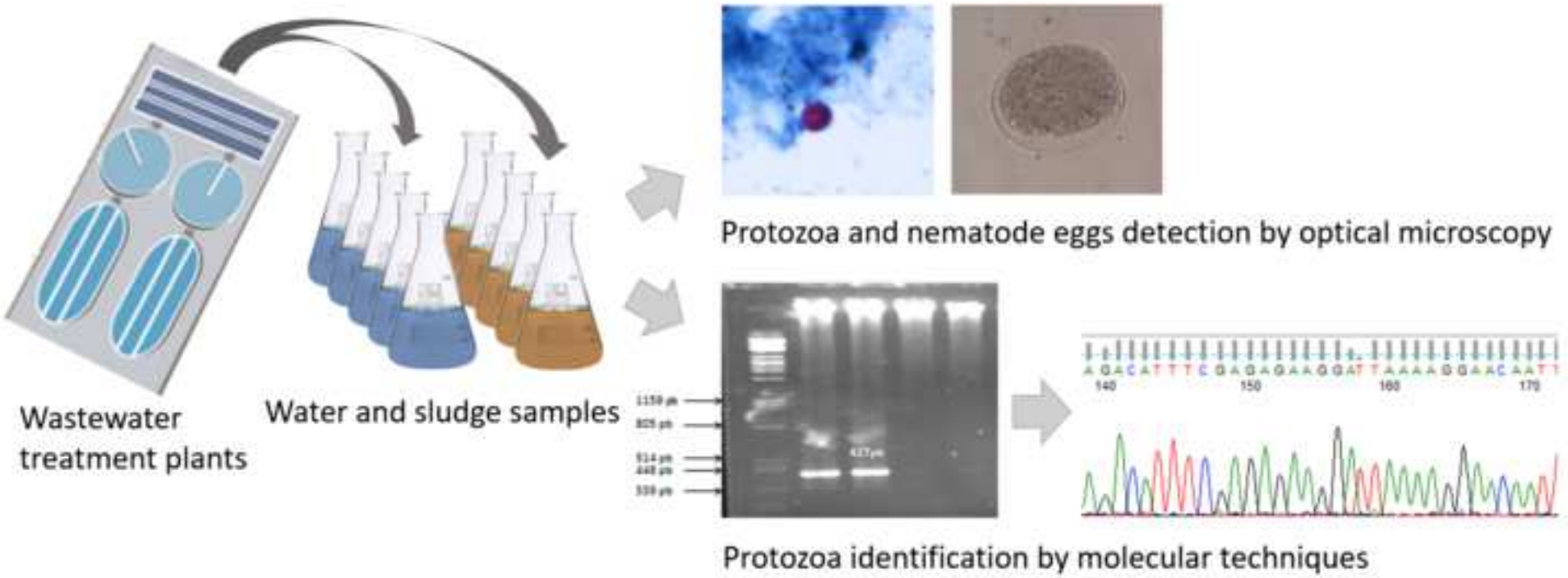
E-mails: mbenito@unizar.es (M. Benito); cmenacho@unizar.es (C. Menacho); pachueca@unizar.es (P. Chueca); mpormad@unizar.es (MP Ormad); pgoni@unizar.es (P. Goñi)

Graphical Abstracts



- Wastewater treatment plants did not remove intestinal protozoa from water or sludge
- *E. histolytica* was found after a tertiary treatment based on aerated lagoons
- *E. histolytica* and *E. moshkovskii* were detected for the first time in Spanish WWTPs
- Protozoa presence in wastewater reflects their infective occurrence in the population
- (Oo)cyst viability studies are needed for effluents and sludge reuse in agriculture

ABSTRACT

Some of the microorganisms present in urban wastewater, which include intestinal protozoa and nematodes, can be pathogenic. Their (oo)cyst and egg transmissible stages are very resistant to environmental stresses and disinfectants and they are therefore difficult to remove. Thus, they can constitute a health risk if water or sludge obtained in the purification of wastewater is reused for agricultural purposes. In this context, the presence of intestinal protozoa and nematodes were studied in influents, effluents and sludge from five wastewater treatment plants (WWTPs) in the north of Spain by optical microscopy and PCR techniques. The removal efficiency of different wastewater treatments was also compared. The presence of protozoa has increased among the population discharging waste to WWTPs in recent years. *Cryptosporidium* spp., *Giardia duodenalis*, *Entamoeba* spp. and nematodes were detected in all of the WWTPs. Indeed, this is the first report of *Entamoeba histolytica* and *Entamoeba moshkovskii* in Spanish WWTPs. The water treatments studied showed different removal efficiencies for each species of intestinal protozoa, with the aerated lagoons providing the best results. (Oo)cysts were also detected in sludge even after aerobic digestion and dehydration. To avoid risks, (oo)cyst viability should be analysed whenever the sludge is to be used as a fertilizer. This study reinforces the necessity of establishing legal limits on the presence of protozoa in WWTP effluents and sludges, especially if reuse is planned. Further studies are necessary for a better understanding of the presence and behaviour of intestinal parasites.

Keywords: Intestinal protozoa; nematode; wastewater treatment plant; water reuse; sludge reuse.

1. INTRODUCTION

Intestinal protozoa and nematodes are diarrhea-causing parasites. Their presence is widespread, although they are more recurrent in developing countries where annually 485,000 deaths – 297,000 being children under five years old – are related to diarrhea caused by contaminated water (World Health Organization, 2018a, 2011). In Spain, *Cryptosporidium* spp. and *Giardia duodenalis* are the most common infective parasites, though *Entamoeba histolytica* and nematode infections have also been recorded, but less frequently (Carranza-Rodríguez et al., 2018; Spanish Microbiological Information System, 2017). As far as we know, *Entamoeba moshkovskii* had never hitherto been reported in Spain.

All these pathogens can cause chronic as well as asymptomatic infections in humans. Moreover, animals can be infected and become a reservoir for most of the pathogens. The existence of healthy carriers, along with underdiagnosed and underreported cases, contributes to the spread of such pathogens into the environment. This situation means that the real infection rates are hidden and the seriousness of the impact on the public is underestimated (World Health Organization, 2011).

The transmissible stages of intestinal parasites, (oo)cysts and eggs, are excreted with faeces. Thus, their presence in wastewater may be expected. However, due to the fact that they are highly resistant to environmental stresses and disinfectants, and that conventional wastewater treatment plants (WWTPs) are not designed to remove them, parasitic protozoa and nematodes are sometimes present in effluents and, consequently, they are usually discharged into water bodies (Mosteo et al., 2013). Hence, they can easily spread through the environment, where they can remain viable for long periods, posing a potential health risk (Ben Ayed et al., 2009).

This situation is of concern, especially when reclaimed water is reused for agricultural irrigation, an increasingly common practice as a strategy for reducing water scarcity. Treated

49 water is also reused for recreational, environmental, urban or industrial purposes, which might
50 also increase potential infection (Ben Ayed et al., 2009; Mosteo et al., 2013). Nevertheless,
51 European regulations on wastewater treatment (*Council Directive 91/271/EEC*) only establish
52 limits for some physicochemical parameters of WWTP effluents. Spanish regulations for the
53 reuse of treated water (*Royal Decree 1620/2007*) limit bacteria and nematode egg
54 concentrations and, in the case of water reuse for agriculture, restrictions are also imposed on
55 *Taenia* spp. eggs. Some countries such as Tunisia have regulations similar to the Spanish as
56 they establish chemical, bacteriological and helminth ova limits for treated water and sludge
57 (Ben Ayed et al., 2009; Khouja et al., 2010). However, other countries contemplate more
58 parameters in their regulations. For example, French regulations include faecal enterococci, F-
59 specific bacteriophages and sulphate-reducing bacteria as indicators of pathogenic bacteria,
60 viruses and protozoan parasites, respectively (Abeledo et al., 2018). In some states of the USA,
61 in Australia, Canada and the UK, regulations include the control of the presence of
62 *Cryptosporidium* in drinking or recreational water (Montemayor et al., 2005).

63 European Union regulations (*Council Directive 98/83/EEC*) concerning water intended for
64 human consumption state that water should not contain any microorganism or parasite that
65 could pose a human health risk. However, *Escherichia coli* is established as a faecal
66 contamination indicator even though the lack of correlation between its presence and the
67 occurrence of parasites has been proved (Tandukar et al., 2018). As an example of this lack of
68 correlation, coliform bacteria are very sensitive to UV (Feitosa et al., 2013) while the eggs of
69 parasites and protozoa are more resistant than bacteria. Indeed, protozoa contamination has
70 already been detected in effluents from drinking water treatment plants in Spain (Castro-
71 Hermida et al., 2010).

72 Sludge obtained as a by-product of urban wastewater treatment can be reused as a soil
73 amendment or fertilizer due to the high content of organically bound nitrogen and

74 phosphorous (Lasheras, 2011), as long as regulations are met. In this case, it should be taken
75 into account that (oo)cysts and eggs – not considered in Spanish regulations (*Order*
76 *AAA/1072/2013*; *Royal Decree 1310/1990*) – removed from water during wastewater
77 treatments are settled in sludge, where they can persist unless the necessary sanitization
78 processes are carried out (Konaté et al., 2013). These processes must ensure the elimination of
79 potentially pathogenic microorganisms that may pose a risk to health.

80 To date, various studies have been carried out on the overall elimination effect of intestinal
81 parasites by purification processes (Amoah et al., 2018; Berglund et al., 2017; Fu et al., 2010;
82 Konaté et al., 2013; Lim et al., 2007; Reinoso et al., 2008). Some of the WWTPs considered
83 here have been previously studied (Mosteo et al., 2013), one of them in considerable detail,
84 revealing the presence of intestinal protozoa and helminth eggs in each of the intermediate
85 processes (Marín et al., 2015). The results of these previous studies suggested that testing
86 different treatments in order to compare their effectiveness for the elimination of these
87 parasites would be interesting, (1) to achieve a better understanding of the interaction and
88 survival capacity of protozoa and nematodes in water and sludge, (2) to enhance parasitic
89 infection risk studies, and (3) to make the necessary adaptations to WWTPs according to the
90 needs of the population.

91 The aim of this work was to identify the parasitic protozoa present in the influents, effluents
92 and sludge from five WWTPs and to compare the removal efficiency of intestinal protozoa and
93 nematode eggs by the different processes carried out in these plants. Additionally, the reuse
94 possibilities of treated water and sludge have been analysed.

95 ## 2. MATERIALS AND METHODS

96 ### 2.1. Collection of wastewater samples

97 Five wastewater treatment plants (WWTP) located in the north of Spain were investigated in
98 order to determine the presence of pathogenic intestinal protozoa and nematode eggs. All the

plants received municipal wastewater and discharged their effluents directly into the Ebro River Basin. One of them (E) had a bath zone downstream. The characteristics of the WWTPs studied are described in Table 1. WWTP A, B and C had been previously studied (Marín et al., 2015; Mosteo et al., 2013) and therefore their evolution over time can be surveyed.

The operational processes of each WWTP are summarized in Figure 1. They include preliminary, primary, secondary and, in some cases, tertiary treatments. The exception to the other treatment plants was WWTP E which did not have any wastewater pre-treatment or primary treatment. Instead, the influent entered directly into an Imhoff tank. This tank consists of an upper chamber where sedimentation takes place, from which inorganic and organic solids slide down sloping walls to an entrance into a lower chamber where sludge is collected and the organic fraction is partially digested under natural environmental conditions (water temperature 7 – 18 ˚C) (Metcalf and Eddy, 1995). This sludge was periodically extracted by a tank truck and transported to another plant for treatment. In WWTPs A and B, the generated sludge was treated in the plant itself by aerobic digestion under mesophilic conditions and afterwards by sludge thickeners. In the digestion under mesophilic conditions, temperatures ranged between 35 °C and 45 °C, with a hydraulic retention time of 12 hours. Every 12 hours, fresh sludge was loaded bringing the temperature down to 35 °C, which subsequently rose to a maximum of 45 °C. In WWTPs C and D, the sludge was only thickened. Sludge from WWTP E was not treated in the plant itself (the degradation in the imhoff tank being considered negligible) so it was not analysed.

Samples were taken in spring 2015 in sterile bottles for further analysis, following the ISO 5667-3:2012 procedure. Five or twenty liters of water, depending on the turbidity, were taken from the influent, effluent and some interesting intermediate stages for each WWTP. These point samples are indicated in Figure 1. Sludge samples were taken according to their

availability and accessibility during the collection day. Five liters of the raw sludge were taken, while 500 g were taken in the case of treated sludge.

2.2. Samples conditioning before parasite analyses

2.2.1. Water

Water samples were filtered through 0.7 µm Millipore® cellulose nitrate membranes. The filters were then washed with Tween 80 0.1 % in the presence of zirconium balls and centrifuged at 1,000 x g for 15 min (Ayres and Mara, 1996). An aliquot of both the elution from the filters and the pellet were analysed by microscopy and molecular techniques.

2.2.2. Sludge

Five liters of sludge samples were centrifuged at 10,000 x g for 20 minutes (Beckman J2-21 centrifuge) in order to perform an initial water-dewatered sludge phase separation. Both phases were collected in sterile bottles separately. The supernatant was centrifuged again in the same conditions so that the separation was more effective. The solid phase from the second centrifugation was added to the first. Both water phases from both centrifugations were filtered as water samples (2.2.1.). The elution from the filters and the dewatered sludge were analysed by microscopy and molecular techniques separately.

2.3. Parasite analyses

2.3.1. Microscopy analyses

The sample filtrates were concentrated by the formalin ethyl-acetate method and observed under an optic microscope to detect the presence of protozoa (oo)cysts and helminth eggs (Young et al., 1979). In addition, smears from all the concentrated samples were stained by modified Ziehl-Neelsen stain to detect coccidian oocysts, such as *Cryptosporidium* oocysts (Henriksen and Pohlenz, 1981). Between three and five smears were observed for each sample.

2.3.2. Molecular biology analyses

PCR techniques were used to identify the presence of *Crysptosporidium* spp.*, Giardia duodenalis* and *Entamoeba* spp. Total DNA extraction was carried out using a commercial kit (Norgen Biotek Corp Stool DNA Isolation Kit, Ontario, Canada). The target genes and protocols used for the PCR are shown in Table 2. A negative control in which DNA was replaced by double-distilled sterile water was included in each reaction. The PCR products were separated by electrophoresis on 1.5% agarose gel and visualized under a UV lamp (Vilbert Lourmat Transluminator) (6x8W-312 nm; power 55W). These PCR products were purified with GFX™ PCR DNA and a Gel Band Purification Kit (GE Healthcare) and directly sequenced in both directions. The nucleotide sequences obtained were analysed by the BioEdit v 7.2.5 program, compared with those registered in the GenBank database using the BLAST tool (Basic Local Alignment Search Tool of the National Centre for Biotechnology Information, NCBI) and registered in GenBank under numbers (MN187542- MN187549).

3. RESULTS

The results obtained are shown in Table 3. *Cryptosporidium* spp. (Table 3) was only found in WWTP C influent (C1) and effluent (C2), and in WWTP E effluent (E4). Surprisingly, it was not identified in the influent of WWTP E. *Cryptosporidium* spp. was detected in the sludge at all of the sampling points in the plants (n = 4). Its detection in the aerobically digested sludge from WWTP A (A4) and B (B4) should be highlighted. *Cryptosporidium* concentration in the sludge was high enough to be detected by microscopy, but species identification was only possible in three positive sludge samples: two from WWTP B3 and one from A4. In all cases, *Cryptosporidium hominis* belonging to subtype IbA10G2 was identified by molecular techniques after the sequence of a *gp60* gene fragment was analysed. The sequence of a fragment of *SSU* gene allowed the identification of *C. parvum* in the WWTP C dewatered sludge sample (C3), although its size was too small for GenBank registration. In the remaining

172 samples, *Cryptosporidium* species identification could not be achieved due to the multiple
173 peaks present in the analysed sequences, which may have been caused by the presence of
174 numerous individuals or low concentrations of the parasite.

175 *Giardia duodenalis* was detected in three WWTP influents (A1, B1 and C1) and only in one
176 WWTP effluent (C2). *G. duodenalis* assemblage B was identified by molecular techniques in C1
177 and its sequence was registered in GenBank, while no cysts were observed by microscopy
178 either in water or in sludge. As with *Cryptosporidium*, sequences of the remaining positive
179 samples could not be obtained for species identification.

180 *Entamoeba histolytica/dispar/moshkovskii* were detected in every water and sludge sample
181 point (Table 3). In order to differentiate the species, and especially to identify the pathogenic
182 *E. histolytica*, molecular techniques were performed, as the three species are morphologically
183 identical when observed by optical microscopy. *Entamoeba dispar* was found in all the water
184 and sludge sample points. *E. moshkovskii* was detected in four out of five influents (A1, B1, C1
185 and D1) and four out of five effluents (A2, C2, D2 and E4). As regards sludge, *E. moshkovskii*
186 was found both in the inlet and outlet of the aerobic digester in WWTPs A (A3 and A4) and B
187 (B3 and B4), in addition to the WWTP D dewatered sludge (D3). *Entamoeba histolytica* was
188 only detected in the influent and effluent of WWTP B (B1 and B2) with a very weak
189 amplification in B1, whereas this was very intense in B2. Identified *E. moshkovskii* sequences
190 from A2, A4, C1 and B3, together with an *E. histolytica* sequence from B2, were registered in
191 GenBank (MN134036).

192 No cestode eggs were found in any sample. Nematode eggs were detected in all of the WWTPs
193 by optical microscopy. Eggs were found in every water and sludge sampling point of the
194 WWTPs A, B and C. Larvae and eggs with larvae inside were observed in A2, B1 and C2. In the
195 WWTP D effluent (D2), small eggs between 10-15 microns were found. In WWTP E, eggs were

196 detected at the Imhoff inlet and outlet (E1 and E2) and in the effluent (E4). Three eggs in A1,
197 B2 and B4 were morphologically compatible with *Ascaris* spp.

198 **4. DISCUSSION**

199 **4.1. Previous studies and prevalence of intestinal protozoa**

200 In the present work, intestinal diarrhea-causing protozoa, including *Giardia* spp.,
201 *Cryptosporidium* spp. and *Entamoeba histolytica/dispar/moshkovskii*, were found in all of the
202 studied WWTPs. Three of these plants were analysed in previous studies (Mosteo et al., 2013;
203 Marín et al., 2015).Samples were taken and analysed every three years (2009, 2012 and 2015)
204 what allows to observe variations in the parasites found in each of the wastewater plants.

205 Mosteo et al. (2013) studied WWTPs A, B and C and did not detect any of these protozoa
206 (Mosteo et al., 2013). In subsequent research, Marín et al. (2015) analysed the bacteria and
207 protozoa presence in every process of WWTP A by molecular biological techniques. They
208 identified shapes morphologically compatible with *Cryptosporidium* spp. oocysts in the
209 influent, the thermophilic aerobic sludge digester inlet and outlet, and in the dewatered
210 sludge. However, PCR results did not confirm their presence. By contrast, *Giardia* spp. cysts
211 were not found by microscopy, but molecular techniques did allow the detection of *Giardia*
212 assemblage B and AI in the coarse solids washing water from the influent primary screening
213 and in the thermophilic aerobic sludge digester outlet, respectively. *Entamoeba* spp. cysts
214 were observed by microscopy in the influent, in the grit removed during the pre-treatment and
215 in the trickling filters biofilm. Despite this, *E. histolytica*, *E. dispar* and *E. moshkovskii* were not
216 identified by PCR and hence the cysts would probably belong to other morphologically similar
217 *Entamoeba* species (Marín et al., 2015).

218 Considering the two previous studies and the present one, WWTPs A, B and C have been taken
219 every three years (2009, 2012 and 2015). Only microscopy analyses were used in the earliest

220 study, while molecular techniques that significantly enhance the sensitivity were used in the
221 subsequent studies. Also, a greater number of PCR techniques were used to analyze the
222 samples taken in 2015, compared to those used with the samples taken in 2012. This may have
223 influenced the finding of more parasitic protozoa in more sampling points in the current study
224 than in the previous ones. However, Marín et al. (2015) used the molecular techniques in the
225 analysis of WWTPA, but they did not find some of the protozoa identified in this study, soit can
226 be inferred that the presence of protozoa must have risen among the population that
227 discharges wastewater to this WWTP.

228 It is worth noting that Castro-Hermida et al. (2008), Galván et al. (2014) and Montemayor et al.
229 (2005) agreed that in Spain a peak of *Cryptosporidium* oocysts is observed in WWTPs during
230 spring, the time of year when samples were taken in this study and the two preceding ones. In
231 addition, an extraordinarily high number of these protozoa infections was detected in Spain
232 during the year 2015 (Table 4), coinciding with the greater degree of detection found in this
233 study.

234 In Spain, the prevalence of *G. duodenalis* is between 1.3 and 8.3 times that of *Cryptosporidium*
235 spp. (Table 4). Nonetheless, these values do not necessarily reflect the real infection rates
236 since many cases are underestimated or asymptomatic (Hamilton et al., 2018; Nasser et al.,
237 2012; World Health Organization, 2011).

238 From the *Entamoeba histolytica/dispar/moshkovskii* complex, only *E. histolytica* is considered
239 pathogenic for humans and is thus notifiable. Its prevalence is very low in Spain (Table 4). *E.*
240 *dispar* is considered a non-pathogenic commensal parasite for human beings, though its
241 pathogenicity has recently been questioned (Oliveira et al., 2015). Meanwhile, the widely
242 distributed *E. moshkovskii* is not considered pathogenic, even though it has occasionally been
243 associated with gastrointestinal and dysentery symptoms (Ali et al., 2003; Heredia et al., 2012).

244 *Cryptosporidium* spp., *Giardia duodenalis* (syn. *G: intestinalis*, *G. lamblia*) and *Entamoeba*
245 *histolytica/dispar/moshkovskii* are intestinal diarrhea-causing protozoa. Consequently, their
246 load in WWTP influents is influenced by the infection and excretion rates of the local human
247 and animal – domestic and wild – population (Hamilton et al., 2018; Nasser, 2016; World
248 Health Organization, 2011, Ben Ayed et al., 2009; Berglund et al., 2017; Fonseca et al., 2016).
249 In the present study, the fact that the content of the wastewater is mainly domestic might be
250 the reason why these protozoa were found. Indeed, several studies have reported a high
251 presence of oocysts in wastewater. Moreover, their ability to maintain their viability for almost
252 one year and their high resistance to conventional water treatments allows *Cryptosporidium* to
253 be preserved in effluents (Abeledo-Lameiro et al., 2018; Castro-Hermida et al., 2010, 2008;
254 Cheng et al., 2009; Montemayor et al., 2005; Nasser, 2016; Ramo et al., 2017). In Spain,
255 average concentrations of 6 – 350 oocysts/L and 1 – 390 oocysts/L in WWTP influents and
256 effluents, respectively, have been reported (Castro-Hermida et al., 2010, 2008; Montemayor et
257 al., 2005; Ramo et al., 2017). Also, *Giardia* cysts are very resistant to environmental stresses,
258 being able to remain viable out of host during at least two months, and they are frequently
259 found in WWTPs (Hamilton et al., 2018; Nasser et al., 2012). Average concentrations of 2 –
260 14400 cysts/L from WWTP influents and 2 – 6000 cysts/L from WWTP effluents have
261 previously been reported in the literature in Spain (Castro-Hermida et al., 2010, 2008; Ramo et
262 al., 2017). In the case of *Entamoeba histolytica/dispar/moshkovskii*, their cyst and trophozoite
263 forms cannot be distinguished by optical microscopy and for that reason *Entamoeba* species
264 have not been differentiated in most research studies (Ben Ayed et al., 2009; Khouja et al.,
265 2010).

266 **4.2. *Cryptosporidium* spp.**

267 *Cryptosporidium* spp. was found in all the sludge sampling points whereas it was not found in
268 water, except for WWTP C. This might be due to the adherence of the oocysts to organic

matter particles, which would enhance their precipitation and concentration in sludge, where they could be detected (Castro-Hermida et al., 2008; Hamilton et al., 2018; Nasser, 2016).

In WWTP C, *Cryptosporidium* spp. was found in the influent and the effluent. The concentration of oocysts in the effluent might depend on the concentration in the influent and the treatments used in the WWTP (Cheng et al., 2009). Possibly, the greater presence of this protozoon was due to the solely domestic origin of the wastewater, in contrast to WWTPs A, B and D, where the sewage is a domestic and industrial mix (agribusiness and food-industries). Moreover, the aerated system of the secondary treatment might have promoted the flotation of the oocysts and their persistence in the effluent (Castro-Hermida et al., 2008).

Low concentrations of oocysts were expected in wastewater, as they were not detected in any water sample except in the case of WWTP C, while they were found in all of the sludge samples. Results showed that the sludge treatments carried out in the WWTPs were not able to remove *Cryptosporidium* oocysts, this being consistent with the findings of other authors (Cheng et al., 2009; Graczyk et al., 2008; Ramo et al., 2017; Rimhanen-Finne et al., 2001). In fact, their presence in the digested and/or dewatered sludge would appear to reflect their capacity to resist the different treatments to which they are submitted. However, to ensure that this is the case , viability studies are needed to avoid the spread of viable *Cryptosporidium* oocysts in the environment, which poses a significant human and animal health risk (Graczyk et al., 2008).

Cryptosporidium hominis subtype IbA10G2 was identified in the WWTP B digester inlet (B3) and in the WWTP A sludge digester outlet (A4). *Cryptoporidium parvum* was identified in the sludge from WWTP C. These species are related to 90 % of *Cryptosporidium* infections, so their occurrence in urban wastewater is not surprising (Alves et al., 2003; Cieloszyk et al., 2012; Goñi et al., 2015). Moreover, the IbA10G2 subtype is the most widespread throughout Europe, as well as in Spain (de Lucio et al., 2016).

294 The simultaneous presence of individuals of different species or genotypes in environmental
295 samples is common and provides multiple peaks in the sequences of the fragments obtained
296 by PCR, which prevent their identification. Another limitation of the PCR technique is the
297 possible presence of organisms that provide nonspecific hybridizations and generate these
298 multiple peaks. On the other hand, the identification of a particular subtype or species of
299 *Cryptosporidium* suggests that this is the majority species or subtype, so the presence of other
300 species or subtypes in very low numbers cannot be excluded.

301 According to Castro-Hermida et al. (2010) in their study in Galicia (Spain), trickling filters are
302 efficient for removing *Cryptosporidium* spp. oocysts from water. However, the results obtained
303 for WWTPs A, B and D agree with those found by Cheng et al. (2009) in their study of an Irish
304 WWTP using trickling filters as the secondary treatment. They found very low concentrations
305 of this protozoon both in the influent and the effluent, but an increased concentration of
306 oocysts in the sludge.

307 It should be noted that WWWTP E is a very small plant with a capacity for 200 equivalent
308 inhabitants; therefore, the presence of parasites in the influent must be lower than that of the
309 other treatment plants, which serve a larger population. It is possible that the trickling filters in
310 WWTP E were not able to remove an undetectable number of *Cryptosporidium* oocysts
311 present in the influent and that afterwards the intermittent sand filter retained the oocysts
312 and concentrated them. Thus, they could be detected in the effluent. Although there is a
313 substantial body of literature that describes the use/efficacy of sand filters in the removal of
314 protozoa and cysts in wastewater treatment, some authors have revealed that the yield is not
315 100%. For example, Leonel et al (2016) reported that the intermittent sand filter removed only
316 38.0 +/- 31.6% of *Giardia* spp. cysts from wastewater, so 93 +/- 92 cysts L^{-1} were identified in
317 the final effluent. In WWTP E, the elimination of *Cryptosporidium* from the effluent becomes
318 more relevant due to the presence of a protected zone declared as “bathing water”

downstream, where outbreaks of gastroenteritis may occur if the concentration of oocysts increases (Castro-Hermida et al., 2010; Hamilton et al., 2018; Hlavsa et al., 2018).

4.3. *Giardia duodenalis*

In contrast to *Cryptosporidium*, found in all the WWTPs, *Giardia* was only detected in three of them (A, B and C). Other authors have reported a greater presence of *Giardia* than *Cryptosporidium* in WWTPs, related to a higher incidence of giardiasis among the population (Table 4) (Castro-Hermida et al., 2010; Cheng et al., 2009; Kitajima et al., 2014a; Ramo et al., 2017).

Giardia duodenalis assemblage B was detected in C1. *Giardia* assemblage B is the cause of the most cases of human giardiasis occurring in Spain (Goñi et al., 2010). Ramo et al. (2017) found this assemblage in their study of 23 WWTPs in Aragón (Spain). In the research of Marín et al. (2015) carried out in WWTP A, *Giardia* assemblage B was identified by molecular techniques in the coarse solids washing water from the influent primary screening and *Giardia* assemblage AI in the thermophilic aerobic sludge digester outlet. To date, *Giardia* Assemblage AI has been recorded as affecting pets and livestock in Spain, but not humans (Gómez-Muñoz et al., 2012). in the remaining positive samples, *Cryptosporidium* and *Giardia* subtypes, species or assemblages could not be identified, probably due to the presence of a mix of different individuals.

The results of the wastewater treatments tested (Table 3) showed that the three serial aerated lagoons – with retention times of 4, 4 and 2.5 days, respectively – (WWTP B) and the serial anaerobic, anoxic and aerobic bioreactors (WWTP C) were effective in removing *G. duodenalis* from wastewater. In the comparative study of Fu et al. (2010), higher *Giardia* cyst removal rates were reported for secondary treatments consisting of an oxidation ditch system and the anaerobic – anoxic – aerobic process, in contrast with the conventional activated sludge treatment. In 2007, Lim et al. proved that the retention of wastewater in ponds and lagoons is

344 the main factor in *Giardia* cyst removal, reaching rates of 96 % when the retention time was
345 between 8 and 24 hours. On the other hand, in WWTP C *G. duodenalis* was removed from
346 water whereas *Cryptosporidium* spp. was not. The different size of cysts and oocysts might
347 justify this result (4 – 6 µm and 8 – 12 µm oocyst and cyst diameters, respectively); oocysts
348 might have remained suspended in water, while cyst removal might have yielded better results
349 thanks to their higher settling velocities (Konaté et al., 2013; World Health Organization, 2011).
350 These results agree with those obtained by other authors since wastewater treatment
351 processes are commonly considered more efficient for *Giardia* removal (Castro-Hermida et al.,
352 2010, 2008; Fu et al., 2010; Kitajima et al., 2014b; Lim et al., 2007; Ramo et al., 2017).

353 Trickling filters were not efficient in removing *Giardia* cysts, which is consistent with the
354 findings of Kitajima et al. (2014) and Castro-Hermida et al. (2008), who even reported that the
355 concentration of cysts in the effluent was significantly higher than in the influent. In any case,
356 *Giardia* cysts were discharged from the WWTP A effluent into the river basin, a fact that should
357 be considered in order to prevent any human health threat if this treated water is to be
358 reused. *Giardia* cysts have already been found in vegetables irrigated with polluted water
359 (Amorós et al., 2010).

360 **4.4. *Entamoeba histolytica/dispar/moshkovskii***

361 *Entamoeba histolytica* was detected in the influent and effluent of WWTP B (B1 and B2). It was
362 not found in any sludge sample here or in any other WWTP. On the one hand, due to the low
363 prevalence of this *Entamoeba* in Spain, the presence of *E. histolytica* in wastewater might
364 suggest that the number of healthy carriers is high enough for the cysts to be detected. This is
365 the first report of the emergence of *E. histolytica* in a Spanish WWTP. Ajonina et al. (2018)
366 reported recently the occurrence of cysts in treated water from a German WWTP. On the
367 other hand, its presence in the effluent discharged into the river basin might raise public
368 health concerns if this water is reclaimed for agriculture (Ben Ayed et al., 2009).

As it was not observed in the sludge, *E. histolytica* might have been collected in the tertiary treatment. However, this would be inconsistent with the research of Khouja et al. (2010), who studied six Tunisian WWTPs and reported that aerated lagoons and waste stabilization ponds are effective in removing *Entamoeba dispar/histolytica* from wastewater.

Entamoeba dispar is a highly prevalent protozoon that was not removed by the treatments in the plants, as shown by the results. Similar findings were obtained by Berglund et al. (2017) while studying the influents and effluents of three Swedish WWTPs in 2011 and 2012. It should be added that the removal rate of this *Entamoeba* is not well known due to the scarcity of *Entamoeba s*pp. prevalence studies carried out with species differentiation (Ben Ayed et al., 2009).

Entamoeba moshkovskii was detected in four out of five influents and effluents, suggesting that the WWTP processes were not able to remove this parasite either. These results were similar to those observed in a WWTP in Colombia, where *E. moshkovskii* has a high presence. However, neither *E. histolytica* nor *E. dispar* were detected in that study despite their high prevalence among the Colombian population (Fonseca et al., 2016). It might be added that in some tropical countries, *E. moshkovskii* has been found in human hosts (Ali et al., 2003), while in Spain, as far as we know, this species has not yet been reported in humans or water. Nevertheless, the ability of *E. moshkovskii* to adapt to the environment as a free-living amoeba may have allowed it to colonize some of the reactors in which processes are carried out and to proliferate in the WWTPs (Fonseca et al., 2016; Heredia et al., 2012). Biofilms containing *E. moshkovskii,* among other microorganisms, could have been formed in the reactors, including the sand filter, in which case the *E. moshkovskii* detected in the effluents would be those released from the biofilms. Therefore, *E. moshkovskii* might not necessarily be present in the influent as it is already present in the WWTP.

A consideration of the results and the different treatments carried out in the WWTPs suggests

394 that *E. moshkovskii* may have also been part of the biofilms originated in the trickling filters of
395 WWTPs A, B and D. There, the increase in the biomass probably promoted the development of
396 this *Entamoeba* and its contribution to the organic matter removal. These biofilms would have
397 accumulated the *Entamoeba* and, when releasing and settling themselves, increased its
398 concentration in the sludge (Marín et al., 2015). This would be the reason why *E. moshkovskii*
399 was detected in A3, A4, B3, B4 and D3. Nonetheless, the three aerated lagoons of WWTP B
400 were effective in removing this species from water or, at least, in lowering its concentration
401 sufficiently so that molecular techniques could not detect its presence, since it was not found
402 in B2. *E. moshkovskii* might have inhabited the reactors of WWTP C and helped the depuration
403 process; the aerated system might have promoted its persistence in water and thus in the
404 effluent (C2), avoiding its sedimentation and further detection in the sludge.

405 **4.5. Helminth eggs**

406 A large number of people and animals are affected by helminths worldwide. However, while
407 these infective helminths are common in tropical and subtropical countries, in areas
408 characterized by poor hygienic conditions, lack of sanitary measures and scarcity of drinking
409 water (Jimenez-Cisneros, 2006; Konaté et al., 2013; World Health Organization, 2018b), most
410 of the reported cases in Spain concern immigrants or travellers from endemic areas (Carranza-
411 Rodríguez et al., 2018). In this study, no cestode or trematode eggs were found, not even in
412 the influents.

413 Being intestinal parasites, nematode eggs are discharged into wastewater and, consequently,
414 they reach WWTPs. Free-living nematodes and their eggs can also be found, which do not
415 constitute a public health problem but instead contribute to the purification of the waters by
416 feeding on bacteria and organic matter (Amoah et al., 2018; Ben Ayed et al., 2009; Konaté et
417 al., 2013). Nematode eggs tend to settle and hence their presence was expected in the sludge.
418 Due to their size (20 - 80 µm in diameter), nematode eggs belong to the suspended solids (SS)

419 fraction and their removal rates depend on their settling velocities, which can be increased
420 when attached to suspended particles. Similarly, SS removal processes - such as filtration,
421 sedimentation and coagulation-flocculation - may also be useful in removing nematode eggs
422 (Amoah et al., 2018; Ben Ayed et al., 2009; Jimenez-Cisneros, 2006; Konaté et al., 2013; World
423 Health Organization, 2011). In this work, nematode eggs were observed in all of the effluents
424 and were present in more sample points than in the previous studies (Marín et al., 2015;
425 Mosteo et al., 2013), suggesting a higher presence of nematodes. The increase in
426 temperatures experienced in recent years may have altered the population of nematodes
427 (Majdi et al., 2019).

428 Comparing the studied WWTPs, WWTP D seems to receive a low concentration of free-living
429 nematodes since they were detected neither in the influent nor in the sludge. Nevertheless,
430 they were detected in the effluent, which could be related to the high algae content present in
431 the second sedimentation process. Eisendle (2009) reported that algae increase the retention
432 capacity of organic matter and nutrients, enhancing the biotic processes, as well as providing
433 habitat for nematodes.

434 It is worth noting that only optical microscopy detection of nematode eggs was carried out and
435 therefore it was not possible to differentiate the species nor the pathogenicity. Nonetheless,
436 three nematode eggs detected in A1, B2 and B4 have a morphology compatible with *Ascaris*
437 spp. eggs. These eggs likely belonged to *A. suum* rather than *A. lumbricoides* because of the
438 latter's low prevalence in Spain. *A. suum* is described as a cause of chronic illness in pigs and its
439 prevalence in Spanish hog farms ranges between 28.7 and 48.8 % (Martínez-Pérez et al., 2017).

440 It has been reported that treatments such as activated sludge, aerated lagoons, stabilization
441 ponds and constructed wetlands are efficient in removing helminth eggs (Ben Ayed et al.,
442 2009; Konaté et al., 2013; Reinoso et al., 2008; Sharafi et al., 2012). This would reinforce the
443 view that those detected might belong to free-living nematodes that inhabit the WWTPs. In

444 any case, if water or sludge reuse is contemplated, disinfection and hygienization treatments
445 should be developed as a precaution. In addition, plant-parasitic nematodes have been
446 reported in reclaimed water and hence they should be controlled and reduced before crop
447 irrigation (Santos et al., 2014).

448 **4.6. Reuse possibilities and health risk**

449 Considering the reuse possibilities of effluents and sludge, four out of the five WWTP effluents
450 (A2, B2, C2 and E4) carried at least one of the pathogenic parasites studied and thus their
451 reuse could pose a health risk depending on the final purpose (agricultural, environmental,
452 industrial, recreational or urban).

453 In the case of WWTP E, the presence of a protected bathing area downstream makes
454 maximizing the water microbiological quality necessary so that bathers would not be exposed
455 to any risk. Only the WWTP D effluent could be safely reused, though more studies about *E.*
456 *moshkovskii* pathogenicity are still needed. Moreover, all of the WWTP sludges were positive
457 for *Cryptosporidium* spp. oocysts and hence their use for agricultural fertilization could pose a
458 risk if the viability of those oocysts is confirmed.

459 It should be taken into account that molecular biology techniques allow these parasites to be
460 detected with a high degree of sensitivity and specificity, but they do not differentiate whether
461 they are viable or not (Marín et al., 2015; Rimhanen-Finne et al., 2001). Therefore, if the
462 treatments inactivated the (oo)cysts and/or eggs, this remained unknown. Given the
463 robustness of (oo)cysts and eggs, appropriate disinfection and sanitization processes (tertiary
464 treatments) should be developed depending on the final uses of the treated water and sludge
465 (Abeledo-Lameiro et al., 2018; Graczyk et al., 2008).

466 The results suggest that tertiary treatments consisting of aerated lagoons (WWTP B) are
467 effective in removing *G. duodenalis* and *E. moshkovskii*, rather than *E. histolytica*. Since the

468 literature shows that solar radiation inactivates (oo)cysts thanks to nucleic acid damage
469 (Hamilton et al., 2018) and that aerated lagoons achieve removal rates of almost 99.9 %
470 (Sharafi et al., 2012), analysing the viability of the parasites found in WWTPs in further studies
471 is recommended so that the resistance of parasites to treatments may be clearly understood.

472 In this context, Nasser (2016) pointed out that UV radiation is the most effective disinfection
473 process to deal with *Cryptosporidium* spp., but other studies claim that it is not able to
474 inactivate helminth eggs (Jimenez-Cisneros, 2006; World Health Organization, 2011). Waste
475 stabilization ponds with retention times longer than 20 days and subsurface flow constructed
476 wetlands have high wastewater (oo)cyst and egg elimination rates thanks to sedimentation
477 and natural inactivation processes (Khouja et al., 2010; Montemayor et al., 2005; Sharafi et al.,
478 2012). Incidentally, Abeledo-Lameiro et al. (2018) suggested using ultrasound irradiation owing
479 to its low cost and efficiency. Graczyk et al. (2008) found that sonication and quicklime
480 stabilization were the most effective sanitization treatments for sludge.

481 The presence of protozoa and nematodes in wastewater reflects the infection rates relative to
482 the populations that surround the WWTPs, suggesting the presence of healthy carriers of some
483 intestinal protozoa that are considered to have low or zero prevalence in the region studied,
484 such as *E. histolytica* and *E. moshkovskii*.

485 The results of this qualitative study show the importance of carrying out future studies that
486 should include quantitative methods such as qPCR and determination of the viability of the
487 protozoa, especially after sludge treatment.

488 **5. CONCLUSIONS**

489 • *Cryptosporidium* spp., *Entamoeba* spp. and nematode eggs were detected in every
490 WWTP studied, suggesting a high resistance of these protozoa and nematode eggs
491 to the water treatments analysed in this work. This is the first report of *E.*

histolytica and *E. moshkovskii* in water from WWTPs in Spain. *Giardia duodenalis* was detected in three out of five WWTPs.

- Trickling filters were more efficient in removing *Cryptosporidium* spp. than *G. duodenalis*. In contrast, serial reactors allowed the flotation of *Cryptosporidium* oocysts but removed *Giardia* from water. Regarding tertiary treatments, the aerated lagoons were efficient in removing *G. duodenalis* and *E. moshkovskii*, but not *E. histolytica*, while the sand filter polluted the effluent instead of removing parasites.
- Four out of the five WWTP effluents carried at least one of the pathogenic parasites studied and thus viability studies are necessary to establish whether they can be reused without a health risk, depending on the final purpose.
- *Cryptosporidium* spp. was detected in the sludge in all the WWTPs, even after aerobic digestion. Therefore, its viability should be considered before deciding on its reuse as agricultural fertilizer or whether soil amendment using sludge should be avoided in order to reduce the spread of *Cryptosporidium* spp. into the environment.

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6. BIBLIOGRAPHY

Abeledo-Lameiro, M.J., Ares-Mazás, E., Goméz-Couso, H., 2018. Use of ultrasound irradiation to inactivate *Cryptosporidium parvum* oocysts in effluents from municipal wastewater treatment plants. Ultrason. Sonochem. 48, 118–126. https://doi.org/10.1016/j.ultsonch.2018.05.013

517 Ajonina, C., Buzie, C., Möller, J., Otterpohl, R., 2018. The detection of *Entamoeba histolytica*
518 and *Toxoplasma gondii* in wastewater. J. Toxicol. Environ. Health-Part A-Current issues
519 81, 1–5. https://doi.org/10.1080/15287394.2017.1392399

520 Ali, I.K.M., Hossain, M.B., Roy, S., Ayeh-Kumi, P.F., Petri, W.A., Haque, R., Clark, C.G., 2003.
521 *Entamoeba moshkovskii* infections in children in Bangladesh. Emerg. Infect. Dis, 9, 580–
522 584. https://doi.org/10.3201/eid0905.020548

523 Alves, M., Xiao, L., Sulaiman, I., Lal, A.A., Matos, O., Antunes, F., 2003. Subgenotype Analysis of
524 *Cryptosporidium* Isolates from Humans , Cattle , and Zoo Ruminants in Portugal. J. Clin.
525 Microbiol. 41, 2744–2747. https://doi.org/10.1128/JCM.41.6.2744

526 Amoah, I.D., Reddy, P., Seidu, R., Stenström, T.A., 2018. Removal of helminth eggs by
527 centralized and decentralized wastewater treatment plants in South Africa and Lesotho :
528 health implications for direct and indirect exposure to the effluents. Environmental Sci.
529 Poll. Res. 25, 12883–12895. https://doi.org/10.1007/s11356-018-1503-7

530 Amorós, I., Alonso, J.L., Cuesta, G., 2010. *Cryptosporidium* oocysts and *Giardia* cysts on salad
531 products irrigated with contaminated water. J. Food Protect. 73, 1138–1140.

532 Ayres, R.M., Mara, D.D., 1996. Analysis of Wastewater for Use in Agriculture: A Laboratory
533 Manual of Parasitological and Bacteriological Techniques. Geneva, Switzerland.

534 Ben Ayed, L., Schijven, J., Alouini, Z., Jemli, M., Sabbahi, S., 2009. Presence of parasitic
535 protozoa and helminth in sewage and efficiency of sewage treatment in Tunisia.
536 Parasitol. Res. 105, 393–406. https://doi.org/10.1007/s00436-009-1396-y

537 Berglund, B., Dienus, O., Sokolova, E., Berglind, E., Matussek, A., Pettersson, T., Lindgren, P.E.,
538 2017. Occurrence and removal efficiency of parasitic protozoa in Swedish wastewater
539 treatment plants. Sci. Total Environ. 598, 821–827.

https://doi.org/10.1016/j.scitotenv.2017.04.015

Carranza-Rodríguez, C., Escamilla-González, M., Fuentes-Corripio, I., Perteguer-Prieto, M.-J., Gárate-Ormaechea, T., Pérez-Arellano, J.-L., 2018. Helmintosis y eosinofilia en España (1990-2015). Enferm. Infecc. Microbiol. Clin. 36, 120–136. https://doi.org/https://doi.org/10.1016/j.eimc.2015.11.019

Castro-Hermida, J.A., García-Presedo, I., Almeida, A., González-Warleta, M., Da Costa, J.M.C., Mezo, M., 2008. Contribution of treated wastewater to the contamination of recreational river areas with *Cryptosporidium* spp. and *Giardia duodenalis*. Wat. Res. 42, 3528–3538. https://doi.org/10.1016/j.watres.2008.05.001

Castro-Hermida, J.A., García-Presedo, I., González-Warleta, M., Mezo, M., 2010. *Cryptosporidium* and *Giardia* detection in water bodies of Galicia, Spain. Wat. Res. 44, 5887–5896. https://doi.org/10.1016/j.watres.2010.07.010

Cheng, H.W.A., Lucy, F.E., Graczyk, T.K., Broaders, M.A., Tamang, L., Connolly, M., 2009. Fate of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and *Giardia duodenalis* cysts during secondary wastewater treatments. Parasitol. Res. 105, 689–696. https://doi.org/10.1007/s00436-009-1440-y

Cieloszyk, J., Goñi, P., García, A., Remacha, M.A., Sánchez, E., Clavel, A., 2012. Two cases of zoonotic cryptosporidiosis in Spain by the unusual species *Cryptosporidium ubiquitum* and *Cryptosporidium felis*. Enferm. Infecc. Microbiol. Clin. 30, 549–551. https://doi.org/10.1016/j.eimc.2012.04.011

Council Directive 91/271/EEC (Official Journal of the European Communities N^{o} L 135, May 30, 1991) Concerning urban waste water treatment, 1991.

Council Directive 98/83/EEC (Official Journal of the European Communities N^{o} L 330, December

563 5, 1998) On the quality of water intended for human consumption, 1998.

564 de Lucio, A., Merino, F.J., Martínez-Ruiz, R., Bailo, B., Aguilera, M., Fuentes, I., Carmena, D.,
565 2016. Molecular genotyping and sub-genotyping of *Cryptosporidium* spp. isolates from
566 symptomatic individuals attending two major public hospitals in Madrid, Spain. Infect.
567 Genet. Evol. 37, 49–56. https://doi.org/10.1016/j.meegid.2015.10.026

568 Eisendle, U., 2009. Distribution patterns of free-living nematodes in differently degraded river
569 reaches of the River Wien, Austria. Nematol. 11, 509–521.
570 https://doi.org/10.1163/138855409X12465362560395

571 Feitosa, R.C., Rosman, P.C.C., Carvalho, J.L.B., Côrtes, M.B.V. and Wasserman, J.C., 2013.
572 Comparative study of fecal bacterial decay models for the simulation of plumes of
573 submarine sewage outfalls. Water Sci. Technol. 68, 622-631

574 Fonseca, J.A., Heredia, R.D., Ortiz, C., Mazo, M., Clavijo-Ramírez, C.A., Lopez, M.C., 2016.
575 Identification of *Entamoeba moshkovskii* in treated waste water used for agriculture.
576 EcoHealth 13, 156–160. https://doi.org/10.1007/s10393-015-1084-6

577 Fu, C.Y., Xie, X., Huang, J.J., Zhang, T., Wu, Q.Y., Chen, J.N., Hu, H.Y., 2010. Monitoring and
578 evaluation of removal of pathogens at municipal wastewater treatment plants. Wat. Sci.
579 Technol. 61, 1589–1599. https://doi.org/10.2166/wst.2010.757

580 Galván, A.L., Magnet, A., Izquierdo, F., Fernández Vadillo, C., Peralta, R.H., Angulo, S., Fenoy, S.,
581 Aguila, C., 2014. A year-long study of *Cryptosporidium* species and subtypes in
582 recreational, drinking and wastewater from the central area of Spain. Sci. Total Environ.
583 468–469, 368–375. https://doi.org/10.1016/j.scitotenv.2013.08.053

584 Gómez-Muñoz, M.T., Cámara-Badenes, C., Martínez-Herrero, M. del C., Dea-Ayuela, M.A.,
585 Pérez-Gracia, M.T., Fernández-Barredo, S., Santín, M., Fayer, R., 2012. Multilocus

586 genotyping of *Giardia duodenalis* in lambs from Spain reveals a high heterogeneity. Res.
587 Vet. Sci. 93, 836–842. https://doi.org/10.1016/j.rvsc.2011.12.012

588 Goñi, P., Aldana, D.E., Clavel, A., Seral, C., Remacha, M.A., Castillo, F.J., 2010. Prevalence of
589 *Giardia duodenalis* assemblage B in humans in Zaragoza and León, Spain. Enferm. Infecc.
590 Microbiol. Clin. 28, 710–712. https://doi.org/10.1016/j.eimc.2010.04.010

591 Goñi, P., Almagro-Nievas, D., Cieloszyk, J., Lóbez, S., Navarro-Marí, J.M., Gutiérrez-Fernández,
592 J., 2015. Cryptosporidiosis outbreak in a child day-care center caused by an unusual
593 *Cryptosporidium hominis* subtype. Enferm. Infecc. Microbiol. Clin. 33, 651–655.
594 https://doi.org/10.1016/j.eimc.2015.02.003

595 Graczyk, T.K., Kacprzak, M., Neczaj, E., Tamang, L., Graczyk, H., Lucy, F.E., Girouard, A.S., 2008.
596 Occurrence of *Cryptosporidium* and *Giardia* in sewage sludge and solid waste landfill
597 leachate and quantitative comparative analysis of sanitization treatments on pathogen
598 inactivation. Environ. Res. 106, 27–33. https://doi.org/10.1016/j.envres.2007.05.005

599 Gutiérrez-Cisneros, M.J., Martín-Rabadán, P., Menchén, L., García-Lechuz, J.M., Fuentes, I.,
600 Gárate, T., Bpuza, E., 2009. Absceso hepático amebiano autóctono en España: ¿una
601 enfermedad emergente? Descripción de 2 nuevos casos clínicos y de una técnica
602 diagnóstica basada en la reacción en cadena de la polimerasa. Enferm. Infecc. Microbiol.
603 Clin. 27, 326–330. https://doi.org/10.1016/j.eimc.2008.06.007

604 Hamilton, K.A., Waso, M., Reyneke, B., Saeidi, N., Levine, A., Lalancette, C., Besner, M.-C.,
605 Khan, W., Ahmed, W., 2018. *Criptosporidium* and *Giardia* in Wastewater and Surface
606 Water Environments. J. Environ. Qual. 47, 1006.
607 https://doi.org/10.2134/jeq2018.04.0132

608 Heredia, R.D., Fonseca, J.A., López, M.C., 2012. *Entamoeba moshkovskii* perspectives of a new
609 agent to be considered in the diagnosis of amebiasis. Acta Trop. 123, 139–145.

https://doi.org/10.1016/j.actatropica.2012.05.012

Henriksen, S.A., Pohlenz, J.F.L., 1981. Staining of cryptosporidia by a modified Ziehl–Neelsen technique. Acta Vet. Scand. 22, 594 –596.

Hlavsa, M.C., Cikesh, B.L., Roberts, V.A., Kahler, A.M., Vigar, M., Hilborn, E.D., Wade, T.J., Roellig, D.M., Murphy, J.L., Xiao, L. Yates, K.M., Kunz, J.M., Arduino, M.J., Reddy, S.C., Fullerton, K.E., Cooley, L.A., Beach, M.J., Hill, V.R., Yoder, J.S., 2018. Outbreaks Associated with Treated Recreational Water – United States, 2000–2014. Morbidity and Mortality Weekly Report. 67 (19), 547-551. https://doi.org/10.15585/mmwr.mm6719a3.

ISO 5667-3:2012, n.d. Water quality - Sampling - Part 3: Guidance on preservation and handling of water samples; Part 13: Guidance of sampling of sludges; Parto 15: Guidance on the preservation and handling of sludge and sediment samples..

Jimenez-Cisneros, B.E., 2006. Helminth ova control in wastewater and sludge for agricultural reuse, in: Grabow, W. (Ed.), Water Reuse New Paradigm towards Integrates Water Resources Management in Encyclopedia of Biological, Physiological and Health Sciences. EOLSS Publishers Co. Ltd UNESCO, pp. 429–449.

Khouja, L.B.A., Cama, V., Xiao, L., 2010. Parasitic contamination in wastewater and sludge samples in Tunisia using three different detection techniques. Parasitol. Res. 107, 109–116. https://doi.org/10.1007/s00436-010-1844-8

Kitajima, M., Haramoto, E., Iker, B.C., Gerba, C.P., 2014a. Occurrence of *Cryptosporidium* , *Giardia* , and *Cyclospora* in in fluent and effluent water at wastewater treatment plants in Arizona. Sci. Total Environ. 484, 129–136. https://doi.org/10.1016/j.scitotenv.2014.03.036

Konaté, Y., Maiga, A.H., Basset, D., Casellas, C., Picot, B., 2013. Parasite removal by waste

633 stabilisation pond in Burkina Faso, accumulation and inactivation in sludge. Ecol. Engin.
634 50, 101–106. https://doi.org/10.1016/j.ecoleng.2012.03.021

635 Lalle, M., Jimenez-Cardosa, E., Cardosa, S.M., Pozio, E., 2005. Genotyping of Giardia duodenalis
636 from humans and dogs from Mexico using a beta-giardina nested polymerase chain
637 reaction assay. J. Parasitol. 91, 203–205. https://doi.org/10.1645/GE-293R

638 Lasheras, A.M., 2011. Criterios de estabilización e higienización de fangos de depuradora, in:
639 Ormad, M.P., Calvo, B. (Eds.), Gestión y Tratamiento de Aguas Residuales. pp. 197–204.

640 Leonel, L. P., Tonetti, A. L., Silva, J. C. P., Nunes, E. A. & Anaruma Filho, F., 2016. Reuse of
641 sewage treated effluent in agricultural practices: an alarming presence of *Giardia* spp.
642 cysts. *Ecol. Eng.* 94, 682–687. https://doi.org/10.1016/j.ecoleng.2016.06.126.

643 Lim, Y.A.L., Wan Hafiz, W.I., Nissapatorn, V., 2007. Reduction of *Cryptosporidium* and *Giardia*
644 by sewage treatment processes. Trop. Biomed. 24, 95–104.
645 https://doi.org/10.1186/1475-2859-9-85

646 Majdi, N., Traunspurger, W., Fueser, H., Gansfort, B., Laffaille, P., Maire, A., 2019. Effects of a
647 broad range of experimental temperatures on the population growth and body-size of
648 five species of free-living nematodes. J. Therm. Biol. 80, 21–36.
649 https://doi.org/10.1016/j.jtherbio.2018.12.010

650 Marín, I., Goñi, P., Lasheras, A.M., Ormad, M.P., 2015. Efficiency of a Spanish wastewater
651 treatment plant for removal potentially pathogens: Characterization of bacteria and
652 protozoa along water and sludge treatment lines. Ecol. Eng. 74, 28–32.
653 https://doi.org/10.1016/j.ecoleng.2014.09.027

654 Martínez-Pérez, J.M., Vandekerckhove, E., Vlaminck, J., Geldhof, P., Martínez-Valladares, M.,
655 2017. Serological detection of *Ascaris* suum at fattening pig farms is linked with

performance and management indices. Vet. Parasitol. 248, 33–38. https://doi.org/10.1016/j.vetpar.2017.10.009

Montemayor, M., Valero, F., Jofre, J., Lucena, F., 2005. Occurrence of Cryptosporidium spp. oocysts in raw and treated sewage and river water in north-eastern Spain. J. Appl. Microbiol. 99, 1455–1462. https://doi.org/10.1111/j.1365-2672.2005.02737.x

Mosteo, R., Ormad, M.P., Goñi, P., Rodríguez-Chueca, J., García, A., Clavel, A., 2013. Identification of pathogen bacteria and protozoa in treated urban wastewaters discharged in the Ebro River (Spain): Water reuse possibilities. Wat. Sci. Tech. 68, 575–583. https://doi.org/10.2166/wst.2013.201

Nasser, A.M., 2016. Removal of Cryptosporidium by wastewater treatment processes: A review. Journal of Wat. Health 14, 1–13. https://doi.org/10.2166/wh.2015.131

Nasser, A.M., Vaizel-Ohayon, D., Aharoni, A., Revhun, M., 2012. Prevalence and fate of *Giardia* cysts in wastewater treatment plants. J. App. Microbiol. 113, 477–484. https://doi.org/10.1111/j.1365-2672.2012.05335.x

Oliveira, F.M.S., Neumann, E., Gomes, M.A., Caliari, M.V., 2015. *Entamoeba dispar* : Could it be pathogenic. Trop. Parasitol. 5, 9–14. https://doi.org/10.4103/2229-5070.149887

Order AAA/1072/2013 (BOE N^{o} 142, June 14, 2013) Concerns of the use of the sludges from wastewater treatment plants in agriculture, 2013. Official Gazette, Spain. (Orden AAA/1072/2013, de 7 de junio, sobre utilización de lodos de depuración en el sector agrario, 2013. Boletín Oficial del Estado, España).

Ramo, A., Cacho, E. Del, Sánchez-acedo, C., Quílez, J., 2017. Occurrence and genetic diversity of *Cryptosporidium* and *Giardia* in urban wastewater treatment plants in north-eastern Spain. Sci. Total Environ. 598, 628–638. https://doi.org/10.1016/j.scitotenv.2017.04.097

679 Reinoso, R., Torres, L.A., Bécares, E., 2008. Efficiency of natural systems for removal of bacteria
680 and pathogenic parasites from wastewater. Sci. Total Environ. 395, 80–86.
681 https://doi.org/10.1016/j.scitotenv.2008.02.039

682 Rimhanen-Finne, R., Ronkainen, P., Hänninen, M.L., 2001. Simultaneous detection of
683 *Cryptosporidium parvum* and *Giardia* in sewage sludge by IC-PCR. J. App. Microbiol. 91,
684 1030–1035. https://doi.org/10.1046/j.1365-2672.2001.01468.x

685 Royal Decree 1310/1990 (BOE N^{o} 262, November 1, 1990) Concerns of the legal regime for the
686 reuse of sludge in agriculture, 1990. Official Gazette, Spain. (Real Decreto 1310/1990, de
687 29 de octubre, por el que se regula la utilización de los lodos de depuración en el sector
688 agrario, 1990. Boletín Oficial del Estado, España)

689 Royal Decree 1620/2007 (BOE N^{o} 294, December 8, 2007) Concerns of the legal regime for the
690 reuse of treated water, 2007. Official Gazette, Spain. (Real Decreto 1620/2007, de 7 de
691 diceimbre, por el que se establece el régimen jurídico de la reutilización de las aguas
692 depuradas. Boletín Oficial del Estado, España)

693 Santos, M.C., Martín, I., Trujillo, E.M., 2014. Nematodes as a factor for consideration in the
694 wastewater treatment and water reuse process. Desal. Wat. T 52, 4715–4720.
695 https://doi.org/10.1080/19443994.2013.815734

696 Sharafi, K., Fazlzadehdavil, M., Pirsaheb, M., Derayat, J., Hazrati, S., 2012. The comparison of
697 parasite eggs and protozoan cysts of urban raw wastewater and efficiency of various
698 wastewater treatment systems to remove them. Ecol. Eng. 44, 244–248.
699 https://doi.org/10.1016/j.ecoleng.2012.03.008

700 Spanish Microbiological Information System. Epidemiology National Centre. Carlos III Health
701 Insitute, 2017. Annual report of the Spanish Microbiological Information System 2016.
702 Madrid. (Sistema de Información Microbiológica. Centro Nacional de Epidemiología.

703 Instituto de Salud Carlos III, 2017. Informe anual del Sistema de Información
704 Microbiológica 2016. Madrid)

705 Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R.H., Trout, J.M., Schantz, P.M., Das, P., Lal, A.A.,
706 Xiao, L., 2003. Triosephosphate Isomerase Gene Characterization and Potential Zoonotic
707 Transmission of *Giardia duodenalis*. Emerg. Infect. Dis. 9, 1444–1452.
708 https://doi.org/10.3201/eid0911.030084

709 Tandukar, S., Scherchand, J.B., Bhandari, D., Sherchan, S.P., Malla, B., Shrestha, R.G.,
710 Haramoto, E., 2018. Presence of human enteric viruses, protozoa and indicators of
711 pathogens in the Bagmati River, Nepal. Pathogens. 7, 38, 1-11.
712 doi:10.3390/pathogens7020038

713 World Health Organization, 2018a. Drinking-water, Fact sheets. Retrieved July 18, 2019 from
714 https://www.who.int/news-room/fact-sheets/detail/drinking-water

715 World Health Organization, 2018b. Soil-transmitted helminth infections, Fact sheets. Retrieved
716 July 18, 2019 from https://www.who.int/news-room/fact-sheets/detail/soil-transmitted-
717 helminth-infections

718 World Health Organization, 2011. Guidelines for Drinking-water Quality, 4th ed. Geneva,
719 Switzerland. https://doi.org/10.1016/S1462-0758(00)00006-6

720 Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A.A., Montali, R.J., Fayer, R., Lal, A.A.,
721 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-Subunit
722 rRNA lene Locus. Appl. Environ. Microbiol. 65, 1578–1583.

723 Young, K.H., Bullock, S.L., Melvin, D.M., Spruill, C.L., 1979. Ethyl acetate as a substitute for
724 diethyl ether in the formalin-ether sedimentation technique. J. Clin. Microbiol. 10, 852-
725 853.

726

727 **Figure 1:** Wastewater (→) and sludge treatment (– – →) process schemes,
728 recirculation flows (- - →) and sample collection points (X). *WWTP*: wastewater
729 treatment plant; *Pret.*: pretreatment comprising grating, grit and grease separation; *T.F.*:
730 trickling filters; *Sed.*: sedimentation; *Aer.D.*: aerobic digester; *C.*: centrifuge; *A.S. pond*:
731 aerated and storm pond; *Ana.*: anaerobic reactor; *Anox.*: anoxic reactor; *Aer.*: aerobic
732 reactor; *S.T.*: sludge thickener.

733

Figure 1

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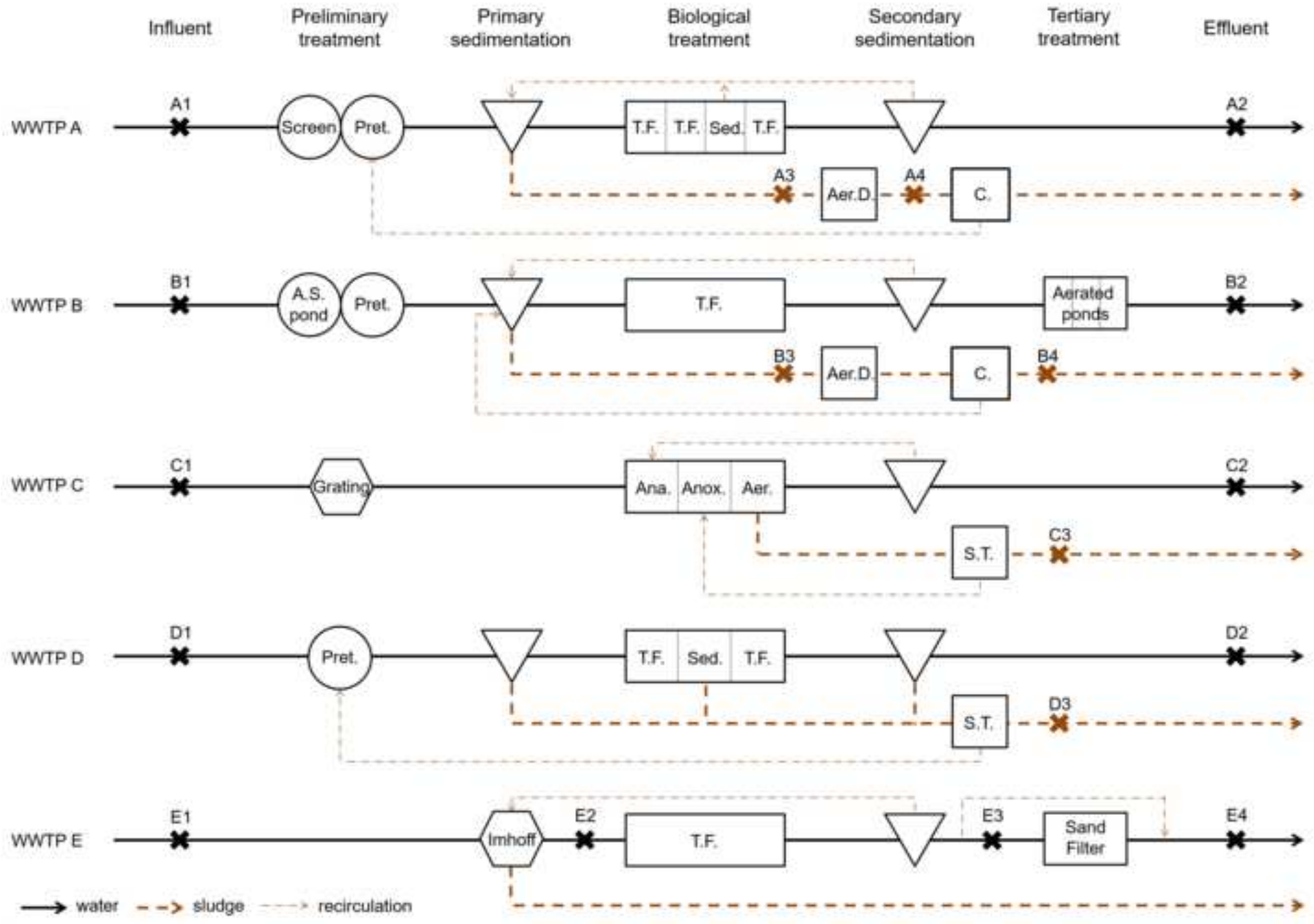

Table 1: Characteristics of the wastewater treatment plants studied

WWTP	Capacity (eq. inhab.)	Discharge (m^3/day)	BOD_5 (kg/day)	Sewage type
A	82500	22100	5200	D + I
B	15900	3000	2600	D + I
C	4300	528	290	D + I
D	19800	1710	1700	D + I
E	200	197	12	D

WWTP: wastewater treatment plant; *Eq. inhab.*: equivalent inhabitants; *BOD_5*: 5-day Biochemical Oxygen Demand; *D*: domestic; *I*: agribusiness or food-industries.

Table 2: Selected genes for protozoa detection by PCR

Specificity	**Gene**	**Reference**
Cryptosporidium spp.	*SSU rRNA*	Xiao et al. (1999)
Cryptosporidium spp.	*gp60*	Alves et al. (2003)
Giardia duodenalis	*tpi*	Sulaiman et al. (2003)
Giardia duodenalis	*B-giardin*	Lalle et al. (2005)
E. dispar	*SSUrADN*	Gutiérrez-Cisneros et al. (2009)
E. histolytica	*SSUrADN*	Gutiérrez-Cisneros et al. (2009)
E. moshkovskii	*SSUrADN*	Ali et al. (2003)

Table 3
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Table 3: Protozoa and nematodes presence in water and sludge samples from the studied wastewater treatment plants

WATER SAMPLES												
	WWTP A		**WWTP B**		**WWTP C**		**WWTP D**		**WWTP E**			
	A1	**A2**	**B1**	**B2**	**C1**	**C2**	**D1**	**D2**	**E1**	**E2**	**E3**	**E4**
***Cryptosporidium* spp.**	Ø	Ø	Ø	Ø	+	+	Ø	Ø	Ø	Ø	Ø	+
G. duodenalis	+	+	+	Ø	+	Ø	Ø	Ø	Ø	Ø	Ø	Ø
E. histolytica	Ø	Ø	+	+	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
E. moshkovskii	+	+	+	Ø	+	+	+	+	Ø	Ø	Ø	+
E. dispar	+	+	+	+	+	+	+	+	+	+	+	+
Nematode eggs	+	+	+	+	+	+	Ø	+	+	+	Ø	+

SLUDGE SAMPLES						
	WWTP A		**WWTP B**		**WWTP C**	**WWTP D**
	A3	**A4**	**B3**	**B4**	**C3**	**D3**
***Cryptosporidium* spp.**	+	+	+	+	+	+
G. duodenalis	Ø	Ø	Ø	Ø	Ø	Ø
E. histolytica	Ø	Ø	Ø	Ø	Ø	Ø
E. moshkovskii	+	+	+	+	Ø	+
E. dispar	+	+	+	+	+	+
Nematode eggs	+	+	+	+	+	Ø

WWTP: wastewater treatment plant. Coloured box with *plus symbol (+):* presence; white box with *null symbol (Ø)*: absence.

Table 4R1
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Table 4: Protozoa infections registered in Spain and in the northern regions of the River Ebro Basin in the annual report of the Spanish Microbiological Information System during the years 2011 – 2016. *Dash symbol (-)*: none cases reported.

	2011	**2012**	**2013**	**2014**	**2015**	**2016**
***Cryptosporidium* spp.**	79	299	107	264	582	180
G. duodenalis	641	942	884	787	778	1067
E. histolytica	9	4	2	12	24	16
***Cryptosporidium* spp.**				**2014**	**2015**	**2016**
Spain				264	582	180
País Vasco				110	250	75
La Rioja				4	140	6
Navarra				73	83	40
Aragón				12	44	8
Cataluña				-	-	15
G. duodenalis				**2014**	**2015**	**2016**
Spain				787	778	1067
País Vasco				189	165	232
La Rioja				52	90	21
Navarra				191	198	155
Aragón				133	148	133
Cataluña				-	-	271
E. histolytica				**2014**	**2015**	**2016**
Spain				12	24	16
País Vasco				2	2	12
La Rioja				-	-	-
Navarra				3	-	-
Aragón				2	-	3
Cataluña				3	19	-

Source: Centro Nacional de Epidemiologia, Instituto de Salud Carlos III. Red Nacional de Vigilancia Epidemiológica

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

***Author Contributions Section**

AUTHOR CONTRIBUTIONS

María Benito: Investigation, Data Curation, Formal analysis; Carmen Menacho: Writing - Original Draft, Writing - Review & Editing, Formal analysis; Patricia Chueca: Investigation, Resources; Peña Ormad: Conceptualization, Methodology, Supervision, Funding acquisition, Writing - Review & Editing; Pilar Goñi: Conceptualization, Methodology, Supervision, Funding acquisition, Writing - Review & Editing, Project administration.