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Additional Information

1 **Simultaneous detection of less frequent waterborne parasitic protozoa in reused**

2 **wastewater using amplicon sequencing and qPCR techniques**

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20 **ABSTRACT**

21 Waterborne parasitic protozoa (WPP) infections have a worldwide distribution and are a
22 source for epidemic and endemic human diseases. Although a variety of protozoa are
23 commonly detected in wastewater and cited as causative agents of outbreaks, effluents
24 from wastewater treatment plants (WWTPs) used for irrigation can contain other
25 pathogenic protozoa that are not currently being controlled. The lack of control on a
26 routine basis using rapid and sensitive methods to detect these parasites in water may keep
27 them under-recognized.

28 This study focused on using molecular tools, 18S rRNA amplicon-based sequencing and
29 qPCR, to characterize WPP distribution in wastewater samples from urban WWTPs used for
30 irrigation. A total of eight wastewater samples (from secondary and tertiary disinfection
31 treatment effluents) were collected. Potentially pathogenic protozoa identified by 18S
32 rRNA sequencing and/or qPCR in the analyzed samples included *Acanthamoeba* spp.,
33 *Blastocystis* sp., *Entamoeba coli, Entamoeba dispar, Entamoeba hartmanni*, *Giardia*
34 *intestinalis* assemblage A and *Toxoplasma gondii* Positive results by qPCR were in non-
35 quantifiable levels. *Blastocystis* sp. was the most represented protozoa among the
36 sequences retrieved from the amplicon sequencing. *Blastocystis* ST1 and ST2 were the most
37 abundant subtypes among the obtained OTUs. Moreover, *Blastocystis* sp. ST3, ST4, ST6 and
38 ST8 were also detected, although in lower abundances. Results of this study showed that
39 WWTP effluents used for irrigation can provide a source of WPP.

40 Keywords: Irrigation water, wastewater, waterborne parasitic protozoa, *Blastocystis*
41 subtypes, qPCR, amplicon sequencing

42 **1. Introduction**

43 Water resources of the European Union are increasingly coming under pressure, leading to
44 water scarcity and a deterioration in water quality (WWAP, 2015). Currently, one-third of
45 the EU territory suffers from water stress all year round due to climate change (Truchado
46 et al., 2021). On a global scale, the volume of treated urban wastewater available for reuse
47 in agriculture can be potentially significant at local level, particularly in arid and semi-arid
48 areas, as it is the Mediterranean zone (Ludwig et al., 2011). In fact, in some European
49 countries, consumption of reused water for agriculture may represent up to 80% of the
50 total water use. The new European regulation on minimum quality requirements (MQR) for
51 water reuse (EU, 2020/741) was launched in May 2020 and describes the directives for the
52 use of reclaimed water for agricultural irrigation. This regulation will be directly applicable
53 in all Member States from 26 June 2023. As stated in this regulation, water reuse is a
54 promising option for many Member States. Still, currently only a small number of them,
55 such as Spain, Cyprus, France or Italy, practice water reuse and have adopted national
56 legislation or standards in that regard (Regulation (EU) 2020/741).

57 Treated wastewater reuse for agricultural irrigation can also promote the circular economy
58 by recovering nutrients from the reclaimed water and applying them to crops (Javanmard
59 et al., 2020). However, it is of the utmost importance to ensure that the use of reclaimed
60 water is safe. In regulation (EU) 2020/741, the concentration of *Escherichia coli* in the
61 different uses of reclaimed water is specified. Only if there is a risk of aerosolization,
62 *Legionella* spp. concentration should also be controlled. Moreover, before a new
63 reclamation facility is put into operation, it should pass a validation monitoring in which

64 the log_{10} reduction of different indicator microorganisms should be controlled, among
65 which the protozoan *Cryptosporidium* can be found (Regulation (EU) 2020/741).

66 Waterborne transmission of pathogenic parasitic protozoa represents one of the most
67 prominent public health issues worldwide. Indeed, waterborne parasitic protozoa (WPP) in
68 wastewater reused for irrigation can lead to contamination of vegetables and to a global
69 public health threat if those vegetables are eaten raw (Amahmid et al., 2021; Javanmard et
70 al., 2018; Spanakos et al., 2015). However, the transmission of parasites through water and
71 the role of emergent and new pathogens are not fully understood. Studies on the
72 occurrence of WPP are fundamental to understanding the epidemiology of waterborne
73 diseases affecting human populations in different geographical regions. Therefore, the
74 development and use of new and rapid approaches are necessary to evaluate the role that
75 irrigation water could have in the transmission of existing, new and emerging pathogens to
76 the human population. Concentration, purification and detection are the three critical
77 steps in all of the methods that have been approved for the routine monitoring of
78 waterborne protozoa (Quintero-Betancourt et al., 2002).

79 Important protozoa prone to water transmission include *Giardia* sp. and *Cryptosporidium*
80 spp., which account for most waterborne outbreaks of pathogenic protozoa reported
81 around the world (Baldursson and Karanis, 2011). Cysts and oocysts of *Giardia* and
82 *Cryptosporidium*, respectively, are especially resistant in the environment and to
83 disinfectants (Erickson and Ortega, 2006; Plutzer et al., 2010). Moreover, low infective
84 doses of cysts and oocysts are enough to cause infection (Almeida et al., 2010).
85 *Cryptosporidium* oocysts and *Giardia* cysts derived from contaminated feces flowing into
86 wastewater treatment systems are often found in the raw wastewater of wastewater
87 treatment plants (WWTPs) in some regions of the world (Cacciò et al., 2003; Alonso et al.,

88 2011; Hatam-Nahavandi et al., 2016; Liu et al., 2011; Guy et al., 2003; Sulaiman et al., 2004).
89 In Spain, *Giardia* cysts and *Cryptosporidium* oocysts have been found in surface waters
90 intended for human or agricultural consumption (Carmena et al., 2007; Castro-Hermida et
91 al., 2009). However, when infectivity was assessed, 57-61% of forms were found to be
92 infective (Castro-Hermida et al., 2010). Moreover, also in Spain, *Cryptosporidium* has been
93 detected in treated wastewater (Abeledo-Lameiro et al., 2018) and both *Cryptosporidium*
94 and *Giardia* were detected in both raw and treated wastewater samples (Domenech et al.,
95 2018; Ramo et al., 2017). (Oo)cysts of both pathogens were found in recycled waters used
96 for irrigation (Spanakos et al., 2015) and in fresh produce irrigated with contaminated
97 water (Amorós et al., 2010; Nguyen et al., 2016.; Utaaker et al., 2017a, 2017b). Consumer
98 safety inherent to the consumption of fresh produce irrigated with wastewater effluents
99 containing pathogens such as *Cryptosporidium* and *Giardia* has been evaluated (Domenech
100 et al., 2018).

101 However, other protozoa, such as *Blastocystis*, *Entamoeba* or different amoebae, have also
102 been described as etiological agents of outbreaks. Nevertheless, insufficient information
103 about them is available, raising the question of whether they are really less frequent in
104 water (Plutzer and Karanis 2016). In fact, in general terms, a small number of WPP are
105 usually present in water, and, although they may be capable of causing disease, they may
106 not be in a sufficient load for detection (Plutzer and Karanis, 2016). The introduction of
107 molecular techniques, particularly those based on the amplification of nucleic acids, has
108 provided researchers with highly sensitive and specific assays for the detection and
109 quantification of protozoa. The use of sequence data generated by massively parallel
110 sequencing, also called ´next-generation sequencing' (NGS), is now commonplace in many
111 fields of biological research (Hino et al., 2016). Studies on molecular detection and

112 characterization of waterborne pathogenic protozoa in wastewater have demonstrated the
113 efficiency of these techniques (Moreno et al., 2018; Xiao and Feng, 2017; Fan, 2021).

114 This study focused on using 18S rRNA amplicon-based sequencing and quantitative PCR
115 (qPCR) techniques to characterize WPP distribution in wastewater samples from urban
116 WWTPs used for irrigation.

117

118 **2. Materials and methods**

119 **2.1. Sampling**

120 Sampling was conducted at two urban WWTPs, WWTP1 and WWTP2, both receiving water
121 from domestic sources close to Valencia city (Spain). In both plants, the first step of water
122 treatment is the screening to remove large objects followed by the secondary treatment,
123 which includes an activated sludge aeration tank (Suppl. Figure 1A). However, only WWTP2
124 includes a treatment with sand filtration in the tertiary stage (Suppl. Figure 1B). Both
125 WWTPs have a final disinfection step using UV lamps, in which the average UV dose is 102
126 mJ/cm^2. A total of eight wastewater samples were collected, six from WWTP1 (3 after
127 secondary treatment and 3 after UV tertiary disinfection treatment) and two from WWTP2
128 (1 after secondary treatment and 1 after tertiary UV disinfection treatment). Sampling was
129 conducted during Spring, and one sampling was performed every week, which consisted of
130 one sample of each type (i.e., after secondary treatment and after tertiary UV disinfection
131 treatment) from one or the other WWTP. Both types of samples from both WWTPs are
132 used for irrigation. A volume of 10 L of each wastewater sample was concentrated through
133 Envirochek HV capsules (1 µm pore size membrane) (Pall Gelman Laboratory, Ann Arbor,
134 MI, USA) following the procedures described in Method 1623.1 of the U.S. Environmental

135 Protection Agency (EPA) (U.S.EPA, 2012). Trapped material on the filter was eluted in 250
136 mL of elution buffer, which was further concentrated by centrifugation at 1,500 x g for 15
137 min, resuspended in 1 mL of PBS and stored at -20°C until DNA extraction was performed.

138 **2.2. DNA extraction**

139 Total DNA was extracted using FastDNA™ SPIN Kit for Soil (MP Biomedicals, Irvine, CA, USA),
140 following the manufacturer's instructions. The homogenization step was carried out in the
141 FastPrep-24® instrument (MP Biomedicals, Irvine, CA, USA) in Lysing Matrix E tube at a
142 speed setting of 6.5 m/s for 120 s. Obtained DNA was eluted in 50 µL of elution buffer and
143 further purified through a PVPP column using OneStep PCR Inhibitor Removal kit (Zymo
144 Research, Orange, CA, USA).

145 **2.3. 18S rRNA amplicon-based sequencing**

146 18S rRNA amplicon-based sequencing was performed as described by Moreno et al. (2018).
147 Illumina sequencing was carried out on a MiSeq platform using the automated cluster
148 generation and paired-end sequencing with dual indexes reads (2 × 300 bp) at FISABIO
149 Sequencing and Bioinformatics Service (Valencia, Spain). The V4 hypervariable region of
150 the 18S rRNA gene was amplified using the primers EUKAF and EUKAR as described by
151 Moreno et al. (2018). Obtained data are deposited in Sequence Read Archive (SRA) under
152 the accession number PRJNA774155.

153 Raw DNA sequencing data was processed using QIIME™ 1.9.1 (http://qiime.org; Caporaso
154 et al., 2010), applying additional scripts available in Microbiome Helper VirtualBox (Comeau
155 et al., 2017) and using the QIIME's open reference script. Operational Taxonomic Units
156 (OTUs) were defined at 97% genetic similarity cut-off. PR2 v4.5 Protist Ribosomal Reference

157 database was used to perform the taxonomic assignment for the eukaryotic microbiome
158 (Gillou et al., 2013).

159 Alpha diversity indices (Observed species, Shannon and Chao1), Good's coverage and
160 rarefaction curves were calculated with subsampled sequencing data (32,462 reads) to
161 reduce the effects of different sampling depths. Mann-Whitney U test was performed using
162 R Software v 4.1.1 (RStudio Team, 2021) to test the significance of diversity differences
163 regarding the wastewater treatment and WWTP ($p < 0.05$). Beta diversity was explored
164 using QIIME, calculating weighted UniFrac distance metrics from the rarefied data and
165 visualized using principal coordinate analysis (PCoA) (Lozupone et al., 2007). The analysis
166 of similarity statistics (ANOSIM) was calculated to test the significance of differences
167 regarding the wastewater treatment and WWTP ($p < 0.05$).

168 Graphic representations were produced using Microsoft Excel 2016 and R Software v 4.1.1
169 (RStudio Team, 2021).

170 **2.4. Phylogenetic analysis of *Blastocystis***

171 *Blastocystis* subtypes were attributed to the OTUs taxonomically assigned to *Blastocystis*
172 using the nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) when the identity
173 percentage was greater than 97%. These sequences, along with reference ones, were
174 aligned using ClustalW algorithm in MEGA11 software (Tamura et al., 2021). A phylogenetic
175 tree was built using the Maximum Likelihood method and Tamura 3-parameter model
176 (Tamura, 1992) in MEGA11 software (Tamura et al., 2021) with 1,000 bootstrap replicates.
177 The analysis involved 74 nucleotide sequences: 53 sample sequences and 21 GenBank
178 reference sequences.

179 **2.4. Quantitative polymerase chain reaction (qPCR)**

180 The WPP screened by qPCR were *Acanthamoeba* spp., *Blastocystis* sp., *Cryptosporidium* 181 *hominis*, *Cryptosporidium parvum*, *Entamoeba histolytica*, *Entamoeba dispar, Giardia* 182 *intestinalis* assemblages A and B, *Naegleria fowleri* and *Toxoplasma gondii* qPCR assays for 183 the detection of *Acanthamoeba*, *Blastocystis*, *Cryptosporidium*, *Giardia* and *Toxoplasma* 184 were performed as previously described (Moreno et al., 2018). *N. fowleri* (Qvarnstrom et 185 al., 2006), *E. histolytica* and *E. dispar* (Berglund et al., 2017) qPCR conditions were adapted 186 for LightCycler 2.0 platform (Roche Applied Science, Spain) in this study as follows. *N.* 187 *fowleri* TaqMan qPCR assay conditions were: initial denaturation at 95 ºC for 10 min, 188 followed by 40 cycles of 10 s denaturation at 95 ºC, annealing at 63 ºC for 10 s and extension 189 at 72 ºC for 7 s; and a final cycle of cooling at 40 ºC for 30 s. *E. histolytica* and *E. dispar* 190 TaqMan qPCR assay conditions were: initial denaturation at 95 ºC for 10 min, followed by 191 40 cycles of 10 s denaturation at 95 ºC, annealing at 60 ºC for 10 s and extension at 72 º C 192 for 7 s; and a final cycle of cooling at 40 ºC for 30 s. Primers sequences and specifications 193 can be found in Supplementary Table 1.

194

195 **3. Results and discussion**

196 Current opinion suggests that molecular techniques are the most promising methods for 197 sensitive, accurate, and simultaneous detection of protozoan parasites compared to 198 conventional staining and microscopy methods, which much benefit the water industry and 199 public health (Fletcher et al., 2012). In this study, the molecular techniques 18S rRNA 200 amplicon-based sequencing and qPCR were used to characterize WPP distribution in 201 wastewater samples from urban WWTPs used for irrigation.

202 After 18S rRNA amplicon-based sequencing, a total of 519,449 sequences were obtained.
203 Once chimeric and low-quality sequences were removed, 459,263 high-quality sequences
204 remained, which were clustered into 2,034 OTUs. All samples were rarefied to an equal
205 sampling depth of 32,462 sequences/sample to make comparisons among them on an
206 equal basis. After rarefaction, the total sequences count was 259,696, which were clustered
207 into 1,636 OTUs (Table 1).

208 Good's coverage values and rarefaction curves were used to estimate how representative
209 the obtained data are of the eukaryotic community. These values ranged from 99.33% to
210 99.63% (Table 2), which meant that almost all the eukaryotic community was revealed in
211 all analyzed samples. Moreover, these results were supported by the rarefaction curves
212 (Figure 1). Observed species and Chao1 alpha diversity indices indicated community
213 richness, and Shannon index was used to reveal community diversity. Although it seemed
214 that the eukaryotic community diversity in the secondary wastewater samples was greater
215 than in the tertiary treatment ones and that both richness and diversity were higher in
216 WWTP1 than in WWTP2, no differences in alpha diversity indices between samples
217 regarding the treatment stage or WWTP were statistically significant ($p > 0.05$). (Suppl.
218 Figures 2, 3). Beta diversity based on weighted UniFrac distance metrics is represented in
219 figure 2. It shows clustering regarding the WWTP where samples were collected but no
220 clustering regarding wastewater treatment, which was confirmed by the ANOSIM test, thus
221 meaning that the eukaryotic community was different in each WWTP, despite the low
222 numbers of analyzed samples, specially from WWTP2 and the difference in sample
223 numbers.

224 The most abundant phyla were Opisthokonta (43.65%), Archaeplastida (10.48%),
225 Stramenopiles (9.79%) and Alveolata (1.09%), which represented 65.01% of the total

226 eukaryotic microbiome (Figure 3). There was a large number of unassigned taxa, as it was
227 the case in other previous studies (Ting et al., 2021), thus suggesting that there is a lack of
228 references in 18S rRNA databases which lead to the non-identification of these taxa and
229 need to be updated.

230 Opisthokonta was also a dominant phylum in wastewater samples in the study carried out
231 by Ting et al. (2021), who also studied urban wastewater samples, but, in their case, along
232 a river, from the inlet, which receives urban wastewater, to the outlet. Although in Ting et
233 al. (2021) study, chytrids and microsporidia were the most abundant Opisthokonta
234 eukaryotes, in the present study these organisms were present in low abundances. In the
235 current study, Opisthokonta phyla was mainly represented by nematodes, such as *Tobrilus*
236 sp.; fungi, such as *Cryptomycotina* sp.; and rotifers such as *Brachionus calyciflorus*.

237 Regarding the potential WPP, *Blastocystis* sp. and *Entamoeba* (*Entamoeba coli*, *E. dispar*
238 and *Entamoeba hartmanni*) were detected by amplicon sequencing in both WWTPs and
239 treatments in the percentages specified in table 3. In general terms, relative abundances
240 of these WPP were higher in samples collected after secondary treatment than those
241 collected after tertiary disinfection treatment, suggesting that this last treatment
242 effectively reduced WPP loads. However, a larger number of samples should be analyzed
243 to confirm these results. Stensvold et al. (2019) identified *Entamoeba* and *Blastocystis* as
244 universal members of the "sewage microbiome". In fact, Zahedi et al. (2019) also detected
245 different species of *Blastocystis* and *Entamoeba* in untreated and in treated wastewater
246 samples using amplicon sequencing. Several waterborne outbreaks caused by *E. histolytica*
247 have been reported (Baldursson and Karanis, 2011; Karanis et al., 2007). Although
248 microscopy is the most common method used to detect *Entamoeba*, it does not allow
249 differentiating between *Entamoeba* species (Ngui et al., 2012). Regarding *Blastocystis*,

250 waterborne outbreaks produced by this protozoan have been only reported in China (Wu
251 et al., 2000) and Italy (Guglielmetti et al., 1989). The development of molecular approaches
252 has increased the detection sensitivity for *Blastocystis*, especially in water samples.

253 By qPCR, the detected potential WPP were *Acanthamoeba* spp., *Blastocystis* sp., *E. dispar*,
254 *G. intestinalis* assemblage A and *T. gondii*, all of them in non-quantifiable levels (Ct>35,00),
255 thus suggesting that there is little human health risk. The protozoa *C. hominis, C. parvum*,
256 *E. histolytica*, *G. intestinalis* assemblage B and *Naegleria* spp. were not detected in any of
257 the samples by qPCR (Table 4). In fact, sequences of these protozoa were neither retrieved
258 by amplicon sequencing. Likewise, *Blastocystis* sp. and *E. dispar* were detected by both
259 molecular techniques. However, *G. intestinalis* assemblage A, *Acanthamoeba* spp. and *T.*
260 *gondii* were only detected by qPCR, thus not recovering any sequence of these taxa after
261 Illumina amplicon sequencing. This could be explained by the fact that these WPP were
262 present in very low concentrations and amplicon-based sequencing has less sensitivity than
263 qPCR technique, especially for low abundant microorganisms (Ahmed et al., 2017).
264 Moreover, the reason of the limited ability to detect *G. duodenalis* by amplicon sequencing
265 remains obscure (Chihi et al., 2022).

266 *G. intestinalis*, along with *Cryptosporidium*, the latter not detected in the analyzed samples,
267 are the most common waterborne infectious agents causing diarrhea and are best known
268 for their potential to cause large waterborne outbreaks of illness (Nguyen et al., 2016;
269 Efstratiou et al., 2017). Both are common water contaminants and have been detected in
270 irrigation water, effluents and biosolids from wastewater treatment plants (Amorós et al.,
271 2010, 2016). The only *G. intestinalis* assemblages capable of not only infecting humans, but
272 also found in numerous other mammals, are assemblages A and B (Monis et al., 2003),
273 which were the ones analyzed in this work by qPCR. *Giardia* has a low infective dose and a

274 marked resistance to environmental and water treatment stresses, which assists their
275 dissemination, and have the potential to be transmitted from non-human to human hosts
276 (zoonosis) and vice versa, enhancing the reservoir of cysts markedly (Smith et al., 2007).

277 *E. histolytica* is a parasitic protozoan capable of causing amoebiasis, which is a type of
278 gastroenteritis (Berglund et al., 2017). Although this species has not been detected by qPCR
279 nor by amplicon metagenomics, other species of *Entamoeba* genus have shown to be
280 present in the analyzed samples: *E. coli*, *E. dispar* and *E. hartmanni*. Although the
281 amoebiasis caused by *E. histolytica* is one of the diseases responsible for most deaths in
282 the world, *E. dispar* has traditionally been considered as non-pathogenic. Still, this view is
283 being re-evaluated (Oliveira et al., 2015). As it occurred in the present work, Berglund et al.
284 (2017) detected *E. dispar* but not *E. histolytica* in the effluent of different Swedish WWTPs,
285 using a qPCR assay which can distinguish between the two species. However, other authors
286 were able to detect *E. histolytica* in influent wastewater samples (Stensvold et al., 2019).
287 Moreover, the 18S rRNA amplicon-based sequencing method used in the current study
288 could also differentiate between *Entamoeba* species, and the less common species *E. coli*
289 and *E. hartmanni* were found in wastewater samples. Cutolo et al. (2012) previously
290 detected *E. coli* in treated wastewater samples. Stensvold et al. (2019) previously detected
291 *E. hartmanni* in influent wastewater samples, however, as far as we are concerned, this is
292 the first time that *E. hartmanni* is detected in treated wastewater samples. Moreover, it
293 should be noted that Stensvold et al. (2019) detected *Entamoeba moshkovskii*, which has
294 the potential to infect humans, in every influent wastewater sample. Conversely, in the
295 current study this protozoan was not detected in any sample, thus suggesting that, in case
296 of being present in the influent samples, it was eliminated during the wastewater
297 treatment process.

298 Regarding free-living amoebae, neither *Acanthamoeba* spp. nor *Naegleria* spp. were
299 detected by amplicon sequencing. However, the former was detected in all wastewater
300 samples by qPCR. In fact, *Acanthamoeba* spp. is a ubiquitous FLA which has been previously
301 found in treated wastewater (Magnet et al., 2012, 2013, Moreno-Mesonero et al., 2017).
302 They are frequently present in urban wastewater samples, since these typically have high
303 concentration of organic matter and bacteria which serve as food (Ramirez et al., 2014).
304 Among FLA, water transmission of pathogenic strains of *Acanthamoeba* spp. is of highly
305 relevant (Plutzer and Karanis 2016).

306 *T. gondii* usually causes asymptomatic infection in immunocompetent individuals, while in
307 immunocompromised people it can induce extraintestinal disease, with involvement of the
308 central nervous system and leading to abortion in pregnant women (Barratt et al., 2010).
309 Oocysts can be encountered in different terrestrial and aquatic systems where they can
310 persist for months (Rousseau et al., 2018). As in the present study, in which *T. gondii* has
311 been only detected by qPCR in two samples (one after secondary and another after tertiary
312 treatments), in Gallas-Lindemann et al. (2013) work, it was detected in a few treated
313 wastewater samples.

314 *Blastocystis* sp. was the most represented pathogenic protozoa after processing the results
315 of 18S rRNA amplicon sequencing. These protozoa are commonly found in the intestinal
316 tract of human beings and a wide range of other mammals, birds, insects and reptiles. It is
317 predominantly transmitted through contaminated food and water as well as close contact
318 with animals (Clark et al., 2013; Parija and Jeremiah 2013). The prevalence of *Blastocystis*
319 in humans varies from 0.5% to 30% in developed countries, and from 30% to 76% in
320 developing countries (Audebert et al., 2016). Moreover, it has been the etiological agent in
321 several reported waterborne outbreaks (Plutzer and Karanis 2016). *Blastocystis* is

322 genetically diverse and at least 17 subtypes (STs) have been described based on the SSU
323 rDNA (Plutzer and Karanis 2016). Moreover, at least eight subtypes (ST1–ST8) are shared
324 by human and non-human hosts, suggesting potential zoonotic and anthroponotic
325 transmission (De la Cruz and Stensvold, 2017). In the present study, *Blastocystis* ST1 and
326 ST2 were the most represented and abundant subtypes among the obtained OTUs.
327 *Blastocystis* subtypes ST3, ST4, ST6 and ST8 were also detected, although in lower
328 percentages (Table 5). *Blastocystis* sequences were deposited in GenBank database under
329 the accession numbers OK598978-OK599030. *Blastocystis* ST1 has been isolated not only
330 in wastewater but also in drinking water distribution systems (Plutzer and Karanis 2016).
331 Banaticla and Rivera (2011) detected *Blastocystis* ST1 and ST2 in both influent and effluent
332 wastewater samples. Javanmard et al. (2019) detected *Blastocystis* subtypes ST2, ST6 and
333 ST8 in treated wastewater used for irrigation. Zahedi et al. (2019) found *Blastocystis* ST1,
334 ST2, ST3, ST4 and ST8 in both influent and effluent wastewater samples and *Blastocystis*
335 ST6 only in influent wastewater. Moreover, Stensvold et al. (2019) found *Blastocystis* ST1,
336 ST2, ST3, ST4, ST8 and ST10 in influent wastewater samples. *Blastocystis* ST3 is the most
337 common associated with illness in human prevalence studies followed by ST1 and ST2 (Tan
338 et al., 2008; Moosavi et al., 2012). Furthermore, the prevalence of ST4 is subject to
339 remarkable variation, being virtually absent in most countries outside of Europe (De la Cruz
340 and Stensvold, 2017). The other found subtypes, ST6 and ST8, are rarely seen in humans,
341 possibly reflecting cases of zoonotic transmission (De la Cruz and Stensvold, 2017).

342 As seen in the phylogenetic tree, all *Blastocystis* OTUs assigned to the same subtype (using
343 nucleotide BLAST tool) clustered together and with the provided reference, thus suggesting
344 that this method of *Blastocystis* subtypes assignment is effective (Figure 4). Besides, used

345 primers target a region of the 18S rRNA gene that would facilitate the detection and
346 identification of *Blastocystis* subtypes in reused waters.

347 **Conclusions**

348 Results of this study showed that WWTP effluents used for irrigation can provide a source
349 of WPP. However, more samples should be analyzed in further studies to obtain more
350 significant results and compare them in statistical terms. Sequences of the WPP *Blastocystis*
351 sp., *E. coli*, *E. dispar* and *E. hartmanni* were retrieved using 18S rRNA amplicon-based
352 sequencing technique in wastewater samples after secondary and tertiary disinfection
353 treatments. The WPP *Acanthamoeba* spp., *Blastocystis* sp., *E. dispar*, *G. intestinalis*
354 assemblage A and *T. gondii* were detected using qPCR technique also in both types of
355 analyzed samples, although in non-quantifiable levels. It should be noted that only DNA
356 was detected, thus we cannot be sure of the viability and infective of the WPP found in this
357 study. Therefore, a positive result does not necessarily mean that there is a human health
358 risk. Regarding *Blastocystis,* the subtypes ST1, ST2, ST3, ST4, ST6 and ST8 could be
359 determined from OTUs and their subsequent analysis. Then, we propose the use of
360 standard identification methods together with 18S rRNA amplicon-based sequencing and
361 qPCR methodologies as a powerful approach to detect simultaneously and specifically the
362 most important WPP present in irrigation waters. This would enable a better
363 microbiological control of wastewater used for irrigation to prevent possible health threats
364 due to the presence of these less frequently studied WPP.

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369 World Joint Programming Initiative (Water JPI) Pilot Call.

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633 **Figure captions:**

634 Figure 1: Rarefaction curves. The x-axis shows the number of sequences per sample and
635 the y-axis shows the observed species (OTUs) at each sampling depth.

636 Figure 2: Two-dimensional principal coordinate analysis (PCoA) plots based on weighted
637 UniFrac distance matrices. Red circles represent wastewater samples taken after secondary
638 treatment. Blue squares represent wastewater samples taken after tertiary disinfection
639 treatment.

640 Figure 3: Relative abundances (%) of the most dominant phyla of the eukaryotic
641 microbiome.

642 Figure 4: Phylogenetic tree of *Blastocystis* OTUs. The percentage of trees in which the
643 associated taxa clustered together is shown next to the branches. ○: *Blastocystis* OTU
644 sequences retrieved in this study ●: *Blastocystis* reference sequences