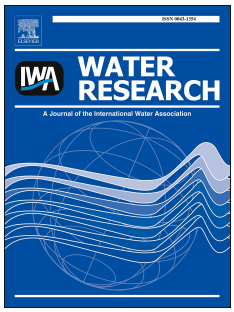
Accepted Manuscript



Identification of eukaryotic microorganisms with 18S rRNA next-generation sequencing in wastewater treatment plants, with a more targeted NGS approach required for *Cryptosporidium* detection

Alireza Zahedi, Telleasha L. Greay, Andrea Paparini, Kathryn L. Linge, Cynthia A. Joll, Una M. Ryan

PII: S0043-1354(19)30349-5

DOI: https://doi.org/10.1016/j.watres.2019.04.041

Reference: WR 14627

To appear in: *Water Research*

Received Date: 24 January 2019

Revised Date: 2 April 2019

Accepted Date: 19 April 2019

Please cite this article as: Zahedi, A., Greay, T.L., Paparini, A., Linge, K.L., Joll, C.A., Ryan, U.M., Identification of eukaryotic microorganisms with 18S rRNA next-generation sequencing in wastewater treatment plants, with a more targeted NGS approach required for *Cryptosporidium* detection, *Water Research* (2019), doi: https://doi.org/10.1016/j.watres.2019.04.041.

WWTPs

- WWTP 1
- WWTP 2
- WWTP 3
- WWTP 4

Eukaryotic 18S NGS

Intestinal parasites detected:

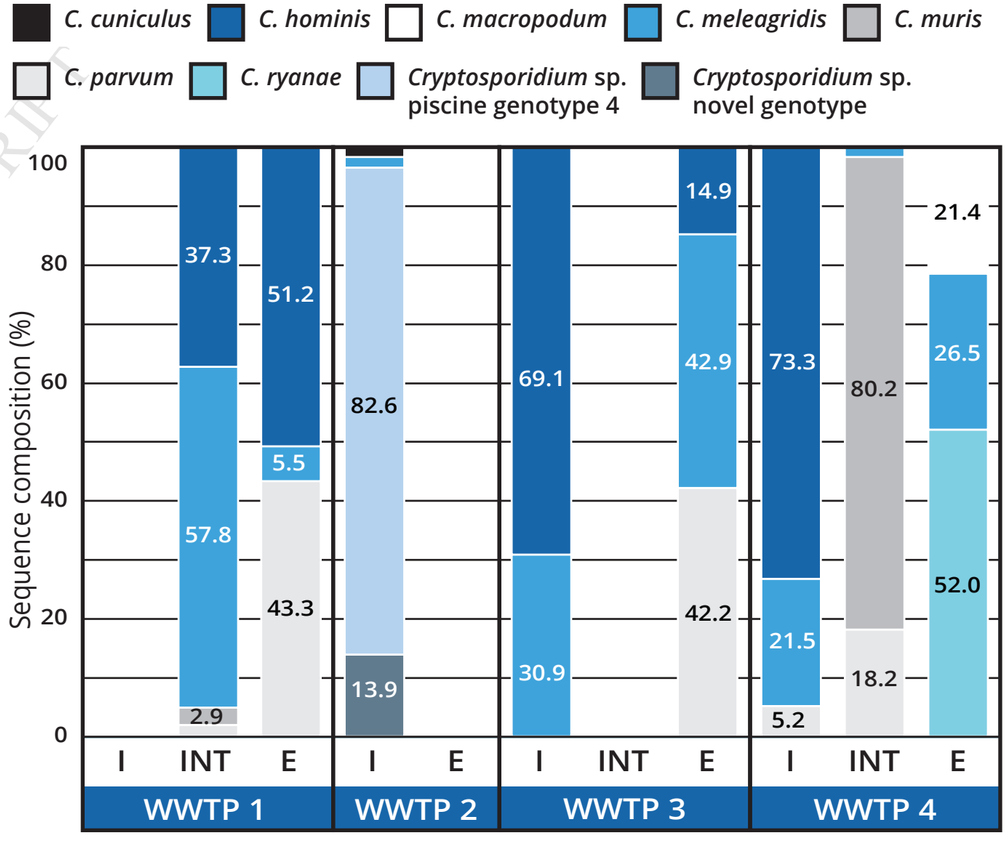
Amoebozoa
Endolimax spp.
Entamoeba spp.
Iodamoeba spp.

Nematoda
Enterobius vermicularis

Sarcomastigophora
Blastocystis sp. subtypes

! *Cryptosporidium* sequences not detected

Cryptosporidium-specific 18S NGS



Identification of eukaryotic microorganisms with 18S rRNA next-generation sequencing in wastewater treatment plants, with a more targeted NGS approach required for *Cryptosporidium* detection

Alireza Zahedi[1,2], Telleasha L Greay[1,2], Andrea Paparini[1], Kathryn L Linge[3,4], Cynthia A Joll[3], Una M Ryan[1*]

[1]Vector and Waterborne Pathogens Research Group, School of Veterinary and Life Sciences, Murdoch University, Perth, Western Australia, Australia

[2]Western Australian State Agricultural Biotechnology Centre, Murdoch University, Perth, Western Australia, Australia

[3]Curtin Water Quality Research Centre, Chemistry, School of Molecular and Life Sciences, Curtin University, GPO Box U1987, Perth, Australia

[4]ChemCentre, PO Box 1250, Perth, Australia

*Correspondence: una.ryan@murdoch.edu.au

Postal address of corresponding author: Veterinary and Life Sciences, 90 South Street, Murdoch University, Murdoch, Western Australia, 6150

Emails:

AZ: a.zahediabdi@murdoch.edu.au

TLG: t.greay@murdoch.edu.au

AP: a.paparini@murdoch.edu.au

KLL: klinge@chemcentre.wa.gov.au

CAJ: c.joll@curtin.edu.au

Abstract

While some microbial eukaryotes can improve effluent quality in wastewater treatment plants (WWTPs), eukaryotic waterborne pathogens are a threat to public health. This study aimed to identify Eukarya, particularly faecal pathogens including *Cryptosporidium*, in different treatment stages (influent, intermediate and effluent) from four WWTPs in Western Australia (WA). Three WWTPs that utilise stabilisation ponds and one WWTP that uses activated sludge (oxidation ditch) treatment technologies were sampled. Eukaryotic 18S rRNA (18S) was targeted in the wastewater samples (n = 26) for next-generation sequencing (NGS), and a mammalian-blocking primer was used to reduce the amplification of mammalian DNA. Overall, bioinformatics analyses revealed 49 eukaryotic phyla in WWTP samples, and three of these phyla contained human intestinal parasites, which were primarily detected in the influent. These human intestinal parasites either had a low percent sequence composition or were not detected in the intermediate and effluent stages and included the amoebozoans *Endolimax* sp., *Entamoeba* sp. and *Iodamoeba* sp., the human pinworm *Enterobius vermicularis* (Nematoda), and *Blastocystis* sp. subtypes (Sarcomastigophora). Six *Blastocystis* subtypes and four *Entamoeba* species were identified by eukaryotic 18S NGS, however, *Cryptosporidium* sp. and *Giardia* sp. were not detected. Real-time polymerase chain reaction (PCR) also failed to detect *Giardia*, but *Cryptosporidium*-specific NGS detected *Cryptosporidium* in all WWTPs, and a total of nine species were identified, including five zoonotic pathogens. Although eukaryotic 18S NGS was able to identify some faecal pathogens, this study has demonstrated that more specific NGS approaches for pathogen detection are more sensitive and should be applied to future wastewater pathogen assessments.

Keywords: Wastewater, *Cryptosporidium*, *Blastocystis*, *Entamoeba*, next-generation sequencing.

1. Introduction

Microbial eukaryotes play important roles in wastewater treatment plants (WWTPs). They contribute to sludge sedimentation and predate upon bacteria, thereby improving effluent quality (Madoni, 2011) and non-predatory eukaryotes, such as Fungi and Rhizaria are thought to play roles in nitrogen removal (Matsunaga et al., 2014). Wastewater is also a major reservoir for eukaryotic human and animal pathogens that can pose severe threats to public health (Maritz et al., 2017). These include helminths such as *Ascaris lumbricoides*, *Ascaris suum* and *Trichuris trichiura*, and protozoans including *Giardia*, *Blastocystis*, *Cryptosporidium*, *Entamoeba* and *Tritrichomonas fetus* (Fletcher et al., 2012; Berglund et al., 2017; Maritz et al., 2017; Amoah et al., 2018). *Cryptosporidium* is a major human enteric pathogen and is problematic for the water industry due to its prevalence, ability to survive in wastewater treatment plants (WWTPs) and its resistance to chlorine disinfection of water supplies (King et al., 2017; Zahedi et al., 2018a). *Giardia* is another enteric parasite that is also prevalent in wastewater and in a recent meta-review was reported in 23/30 (77%) WWTPs analysed (Nasser et al., 2012).

Eukaryotic communities in WWTPs have been investigated using morphological techniques but have not been well-characterised using molecular tools (Matsunaga et al., 2014). Next-generation sequencing (NGS) technologies have revolutionised our ability to survey microbial communities in wastewater, and while they have been widely used to analyse bacterial communities in WWTPs, relatively few studies have been conducted on eukaryotic communities (Matsunaga et al., 2014).

Another knowledge gap is eukaryotic population dynamics in rural WWTPs. Urban WWTPs generally use costly treatment techniques including reverse osmosis membranes, UV and ozone in combination with activated sludge or membrane bioreactors (Rajasulochana and Preethy, 2016; Garrido-Cardenas et al., 2017; King et al., 2017). However, rural WWTPs

generally utilise simple, non-mechanical waste stabilisation ponds (WSPs), as they are cheap to run and maintain, but relatively little is known about the ecology of eukaryotes in WSPs (Eland et al., 2018). Similarly, the eukaryotic ecology of oxidation ditch WWTPs, which are modified mixed activated sludge systems (Xu et al., 2017), has not been well characterised.

The primary aim of the present study was to screen three WSPs and an oxidation ditch WWTP in Western Australia (WA) for intestinal parasites using eukaryotic 18S rRNA (18S) NGS.

2. Methods

2.1 Study sites and sample collection

Wastewater samples (100 ml) (n = 26) were collected from three WSPs (WWTPs 1, 2 and 3) and an oxidation ditch WWTP (WWTP 4) that primarily treat household sewage in WA (Table 1 and Figure 1). Treated wastewater from WWTP 1 is chlorinated and used to irrigate a number of public spaces, including a golf course and sporting ovals. At the time of the study, WWTP 2 was stored after treatment, with intermittent release to a nearby river when required. However, the treated wastewater from WWTP 2 is now filtered, chlorinated and used for irrigation of public open spaces. WWTP 3 water is used by a nearby industrial zone as process water, and WWTP 4 treated wastewater is used for irrigation of a local golf course, after groundwater infiltration. Samples were collected in February, July and September in 2015 and covered two seasons for each site. Samples were collected from WWTP 1 during the wet and dry season, while samples from WWTPs 2, 3, and 4 were collected during summer and winter (Table 1). Wastewater samples were also collected at different treatment stages (influent, intermediate and effluent) during summer and winter (or dry and wet seasons for WWTP 1

samples) (Table 1). The wastewater samples were collected in 1 L sterile containers that were treated with chlorine and rinsed with the sample before filling. Samples were kept cool in an ice box during transport back to the laboratory, and then stored at 4 °C until required.

2.2 DNA isolation

Each wastewater sample (100 mL) was filtered through a sterile 0.2 µm Sterivex filter (Millipore, USA) and genomic DNA (gDNA) was extracted from the filters using a PowerWater Sterivex DNA Isolation Kit (MOBIO Laboratories, California, USA). Extraction reagent blank controls (ExCs; n = 6) were included alongside each batch of gDNA extractions. Purified DNA was stored at -20 °C prior to molecular analyses.

2.3 Next-generation sequencing library preparation

For NGS library preparation and sequencing, the 16S metagenomic sequencing library preparation protocol from Illumina (Part # 15044223 Rev. B; Illumina, USA) was followed, with only minor modifications to the first stage PCRs. The hypervariable 9 (V9) region of the eukaryotic 18S and a *Cryptosporidium*-specific region of 18S were amplified with primers outlined in Table 2, using 2 µl of template DNA (out of a total of 50 µl); these primers were modified to include Illumina MiSeq adapter sequences (Part # 15044223 Rev. B; Illumina, USA). Amplification of 18S V9 with the Euk1391F/EukBr primers was carried out using conventional PCR as per the 18S amplification protocol available from the Earth Microbiome Project (http://www.earthmicrobiome.org). A mammalian blocking primer (Mammal_block_I-short_1391f) (www.earthmicrobiome.org/protocols-and-standards/18s/) was used at a final concentration of 1.6 µM to reduce amplification of mammalian DNA. The *Cryptosporidium*-

specific conventional PCR was conducted using primers described by Morgan et al. (1997), following the amplification protocol described by Paparini et al. (2015). The libraries were sequenced on the Illumina Miseq platform (San Diego, CA, USA), with v2 sequencing chemistry for the eukaryotic 18S NGS and v3 chemistry for *Cryptosporidium*-specific NGS.

2.4 18S Bioinformatic analysis

NGS data pre-processing steps, which included paired-end read merging, primer trimming, quality filtering and singleton removal were performed using USEARCH v10.0 (Edgar, 2010). Sequences were then denoised into zero-radius operational taxonomic units (ZOTUs) and chimeras were filtered with UNOISE3 (Edgar, 2016). Taxonomic assignment of the ZOTUs was performed in QIIME 2 v2018.2 (Caporaso et al., 2010, https://qiime2.org) using the QIIME 2 feature classifier plugin (Bokulich et al, 2018) and the SILVA v128 sequence database (Quast et al., 2013). The sequences were also BLAST searched against the National Center for Biotechnology Information (NCBI) non-redundant nucleotide (nr/nt) database to determine taxonomy at the species level. To control for 18S sequence laboratory contaminants and cross-contamination, the proportion of reads for each ZOTU identified in the no template controls (NTCs) were bioinformatically removed from the respective ZOTU sequences in the samples and ExCs. Similarly, the proportion of reads for each ZOTU in the ExCs were bioinformatically removed from the respective ZOTU sequences in the samples that the ExCs were extracted alongside.

2.5 *Giardia*-specific PCR

All samples were screened for the presence of *Giardia* at the glutamate dehydrogenase (*gdh*) locus using a quantitative PCR (qPCR) as previously described (Yang et al., 2014). A spike analysis (addition of 0.5 μL of positive control DNA into each sample) at the *gdh* locus by qPCR was conducted on randomly selected negative samples from each group of DNA extractions to determine if negative results were due to PCR inhibition, by comparing the cycle threshold (*Ct*) of the spike and the positive control (both with the same amount of DNA).

2.6 *Cryptosporidium* 18S PCR amplification and Sanger sequencing analysis

Due to its importance as a pathogen in wastewater (Zahedi et al., 2018a), *Cryptosporidium*-specific 18S NGS was conducted to identify any *Cryptosporidium* sequences in the samples. All WWTP samples (n = 26) were screened for the presence of *Cryptosporidium* 18S using nested primers that amplify ~825 bp products, as previously described (Xiao et al., 1999). The amplified DNA from secondary PCRs were separated by gel electrophoresis and purified for Sanger sequencing using an in-house filter tip method (Yang et al., 2013). Purified PCR products were sequenced independently on an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions. Sanger sequencing chromatogram files were imported into Geneious Pro 8.1.6 (Kearse et al., 2012), edited, analysed and aligned with reference sequences from GenBank using Clustal W (http://www.clustalw.genome.jp). NTCs were included alongside all PCRs to detect contamination.

3. Results

3.1 Next-generation sequencing library summary

175

176 Overall, the total number of raw (unprocessed) paired-end sequences obtained for
177 eukaryotic 18S and *Cryptosporidium*-specific 18S NGS were ~4.4 million and ~305,000,
178 respectively (Table 3). After the pre-processing steps (merging, quality filtering, and singleton
179 and chimera removal), potential contaminant ZOTU removal (for eukaryotic 18S NGS only),
180 and non-specific taxa removal (e.g. bacterial 16S sequences), there were ~1.5 million (average
181 41,434) and ~20,000 (average 1,649) sequences for eukaryotic 18S and *Cryptosporidium*-
182 specific 18S NGS, respectively (Table 3).

183

184 **3.2 Eukaryotic 18S NGS**

185

186 **3.2.1 Composition of eukaryotic phyla**

187

188 A total of 3,665 ZOTUs were obtained for eukaryotic 18S NGS. The Euk1391F/EukBr
189 primers used exhibited significant cross-reaction with bacteria and archaea, with ~40% of the
190 reads assigned to bacterial or archaeal 16S. Excluding bacterial and archaeal 16S, 1,598 ZOTUs
191 were assigned to the domain Eukarya, while 38 ZOTUs were not assigned to Bacteria, Archaea
192 or Eukarya. Adequate sequencing depth for eukaryotic 18S NGS was obtained for 17/26 samples
193 at ~30,000 sequences (Figure 2). At least 49 eukaryotic phyla were detected and 17.3% of 18S
194 sequences were not assigned taxonomy at the phylum level (the most specific taxonomy was
195 designated as Alveolata, Eukaryota and Stramenopiles and sequences that were more generally
196 assigned to SAR (Stramenopiles, Alveolata and Rhizaria) in Supplementary File B.1). The most
197 dominant phyla that had sequence compositions of >10% included Chlorophyta in the
198 intermediate (61.1%) and effluent (53.2%) samples for WWTP 1, influent for WWTP 2 (16.1%)
199 and WWTP 4 (48.1%), and in the intermediate for WWTP 3 (25.6%); Choanozoa in the influent

for WWTP 1 (16.1%), WWTP 3 (14.8%) and WWTP 4 (12.8%), and intermediate for WWTP 4 (20.8%); Ciliophora in the influent (11.7%) and effluent (39.4%) for WWTP 2, effluent for WWTP 3 (22.9%), and intermediate (35.2%) and effluent (51.1%) for WWTP 4; and Euglenozoa in the influent (44.6%) and effluent (31.3%) for WWTP 2, intermediate (19.5%) and effluent (37.6%) for WWTP 3, and effluent for WWTP 4 (21.4%) (Figure 3). More specific levels of classification with the top BLAST hit GenBank® accession numbers and percent similarities for each ZOTU are provided in Supplementary File B.1. The most abundant 18S sequences in the ExCs and NTCs were identified as alveolates, fungi (Ascomycota and Basidomycota) and green algae (Charophyta) (Supplementary File B.1).

3.2.2 Prevalence and composition of intestinal parasites

Intestinal parasites were identified from the genera *Blastocystis* (16/26), *Endolimax* (6/26), *Entamoeba* (8/26), *Enterobius* (1/26) and *Iodamoeba* (2/26) in WWTP samples (Supplementary File B.1). One *Blastocystis* sequence was detected in one ExC, therefore samples that contained only one intestinal parasite sequence were considered to be negative. The intestinal parasite 18S sequence abundance was overall highest in the influent, and lowest (≤0.2%) or not detected in the intermediate and effluent stages (Table 4).

All *Blastocystis* sp. subtypes (STs) 1-4, 6, 8, and potentially two novel genotypes (*Blastocystis* sp. ZOTU 1064 and ZOTU 1314) were identified in the influent for WWTP 3 and WWTP 4, with percent sequence compositions ranging from <0.1% for *Blastocystis* ST6 to 7.9% for *Blastocystis* sp. ST1 in WWTP 3, and <0.1% for *Blastocystis* sp. ST6, *Blastocystis* sp. ZOTU 1064 and ZOTU 1314 to 2.6% for *Blastocystis* sp. ST1 in WWTP 4. All STs and genotypes except for *Blastocystis* sp. ZOTU 1314 were detected in the influent for WWTP 1,

224 which similar to WWTP 3 and WWTP 4, had the highest percent sequence composition in the
225 influent for *Blastocystis* sp. ST1 (6.4%).

226 For WWTP 1, all *Entamoeba* species and genotypes were identified, except for the
227 potentially novel genotype *Entamoeba* sp. ZOTU 1622. The species with the highest percent
228 sequence composition in WWTP 1 influent was *Entamoeba coli* (2.2%). For WWTP 3 and
229 WWTP 4, all *Entamoeba* species and genotypes were detected except for *Entamoeba polecki*.
230 Like WWTP 1, the *Entamoeba* species with the highest sequence composition in WWTP 3
231 influent was *Ent. coli* (0.7%). In WWTP 4 influent, the potentially novel *Entamoeba* sp. ZOTU
232 405 had the highest sequence composition (2.9%). No *Entamoeba* species were detected in
233 WWTP 2.

234 21 ZOTUs were assigned to the genus *Endolimax* and the highest similarity was 99.1% to
235 *Endolimax nana* (AF149916), and only 12/21 ZOTUs had ≥94.0% sequence identity to *End.*
236 *nana*. *Endolimax* sp. were detected in WWTP 1, WWTP 3 and WWTP 4, and the percent
237 composition was highest in the influent (0.1-0.7%) (Table 4). *Iodamoeba* sequences 100%
238 similar to *Iodamoeba* sp. (JN635741) were rare (<0.1% percent sequence composition of
239 positive samples), but were found in the influent of WWTP 1 and WWTP 4, and a potentially
240 novel *Iodamoeba* sp. genotype that was 99.1% similar to the same *Iodamoeba* sp. isolate
241 (JN635741) was also found in WWTP 1 (0.1% sequence composition) (Table 4).

242 Sequences from helminths (e.g. *Ascaris lumbricoides*, *Ascaris suum* and *Trichuris*
243 *trichiura*) that are usually common in WWTPs were not detected, but human pinworm
244 (*Enterobius vermicularis*) was detected in the influent of WWTP 1 (1.8% sequence
245 composition). Sequences from zoonotic protists such as *Toxoplasma gondii*, *Tritrichomonas*
246 *fetus*, *Cryptosporidium* sp. and *Giardia* sp. were not detected with eukaryotic 18S NGS.
247 Although *Giardia* sp. was not detected in the wastewater samples using *Giardia*-specific qPCR,
248 *Cryptosporidium* sp. were identified using the *Cryptosporidium*-specific 18S NGS assay.

3.2.3 Prevalence and composition of eukaryotic bioindicators

Bacterivorous eukaryotes were detected in all WWTPs, predominantly in the effluent (8.8% in WWTP 1 – 51.1% in WWTP 4), the influent of WWTP 2 (11.7%) and intermediate of WWTP 4 (35.2%). In general, fungal communities were present at very low levels across the WWTPs, with the exception of the influents (15.8% in WWTP 1, up to 12.8% in WWTP 3 and up to 9.1% in WWTP 4) and the intermediate stages of WWTP 3 (8.1%) and WWTP 4 (5.6%). Green algae were detected in all WWTPs with the highest levels in WWTP1 effluent (53.2%), WWTP 2 and 4 influents (16.1 and 48.1% respectively) and WWTP 3 intermediate stage (25.6%). Rotifers were also present across all WWTPs with the highest prevalence in WWTP 3 effluent (27.9%) (Table 5).

3.3 *Cryptosporidium* sp.

Of the 26 samples, a *Cryptosporidium*-specific 18S nested PCR produced amplicons with the correct band size (confirmed by gel electrophoresis) for 20 samples, and Sanger sequencing identified *Cryptosporidium hominis* in one sample (WWTP 4-4). The remaining 19 samples produced either mixed chromatograms or non-specific sequences (e.g. *Colpodella* sp., which were also detected with eukaryotic 18S NGS). The *Cryptosporidium*-specific 18S NGS analysis identified a total of nine *Cryptosporidium* species across the four WWTPs: *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Cryptosporidium meleagridis*, *Cryptosporidium muris*, *Cryptosporidium cuniculus*, *Cryptosporidium macropodum*, *Cryptosporidium ryanae*, *Cryptosporidium* sp. piscine genotype 4 and a potentially novel *Cryptosporidium* sp. piscine genotype (Figure 4 and Supplementary File B.1). The number of *Cryptosporidium* species in

274 individual WWTPs ranged from 3 to 6. The highest percent sequence composition was for *C.*
275 *hominis* (30.4%, present in 6 samples), which was detected in WWTP 1, WWTP 3 and WWTP
276 4, but not in WWTP 2. As with *C. hominis*, *C. parvum* was only present in WWTP 1, WWTP 3
277 and WWTP 4, and had a lower sequence composition (7.6%). *Cryptosporidium meleagridis* was
278 identified in all four WWTPs (22.8% sequence composition, present in 9 samples), and
279 accounted for 57.8% of *Cryptosporidium*-specific reads in post-maturation pond 1 from WWTP
280 1. *Cryptosporidium muris* was detected in WWTP 1 and WWTP 4 (intermediate samples). The
281 remaining *Cryptosporidium* species were detected in WWTP 2 (*C. cuniculus*, *Cryptosporidium*
282 sp. piscine genotype 4 and *Cryptosporidium* sp. novel piscine genotype in the influent) and
283 WWTP 4 (*C. macropodum* and *C. ryanae* in the effluent).

284 Nucleotide sequences reported in this paper are available in the GenBank database under
285 accession numbers MH623043-623073 and MH979339-979398.

286

287 ## 4. Discussion

288

289 As expected, the eukaryotic 18S NGS assay detected intestinal parasites primarily in the
290 influent, and included the amoebozoans *Endolimax* sp., *Entamoeba* sp. and *Iodamoeba* sp., the
291 human pinworm *Enterobius vermicularis*, and the protozoan *Blastocystis*. Currently, 17
292 *Blastocystis* STs have been described (Alfellani et al., 2013); ST1 and ST3 are the most
293 prevalent STs in Australia (Stensvold et al., 2007), and ST1-ST10 and ST12 infect humans
294 (Forsell et al., 2012; Ramírez et al., 2016). *Blastocystis* infections are usually asymptomatic but
295 can cause gastrointestinal symptoms and are associated with irritable bowel and chronic
296 abdominal pain (Azizian et al., 2017; Toro Monjaraz et al., 2017). There are only a few reports
297 of *Blastocystis* STs in WWTPs (Zaman et al., 1994; Maritz et al., 2017) and very little is known
298 about their prevalence and survival in WWTPs. The genus *Entamoeba* consists of both free-

299 living and parasitic species and has been frequently reported in both untreated and treated
300 wastewater (Cifuentes et al., 1994; Ben Ayed et al., 2009; Fonseca et al., 2016). Four species of
301 *Entamoeba* were identified in the present study; *Ent. coli* and *Ent. polecki* had the highest
302 sequence compositions (2.2% and 0.8%, respectively), whereas *Ent. hartmanni* and *Ent.*
303 *moshkovskii* were rare (≤0.2%). These parasites colonise human intestines and have been
304 considered as non-pathogenic (Clark and Diamond, 1991), but recent reports have found that
305 *Ent. moshkovskii* and *Ent. nuttalli* can cause diarrhoea in humans (Shimokawa et al., 2012;
306 Levecke et al., 2015). *Entamoeba histolytica* is a major cause of diarrhoeal disease that is
307 estimated to affect about 50 million people annually (Fotedar et al., 2007), but unfortunately,
308 *Ent. histolytica* cannot be differentiated from *Entamoeba dispar* and *Entamoeba nuttalli* at the
309 18S V9 region. *Entamoeba* sp. ZOTU 832 was 100% identical to *Ent. histolytica*, *Ent. dispar*
310 and *Ent. nuttalli* sequences on GenBank (Table 4). The health significance of *Endolimax* sp.
311 detected in the present study, which were most similar to the human intestinal parasite
312 *Endolimax nana*, is unknown. *Endolimax* sp. have been reported in a variety of amphibians,
313 birds, fish, mammals and reptiles, and like *Blastocystis* STs and *Entamoeba* sp., can be
314 transmitted by faecal-oral contamination of food and water (Poulson and Stensvold, 2016).
315 Although *Endolimax* has been associated with diarrhoea (Graczyk et al., 2005; Shah et al.,
316 2012), there is currently no consensus on the pathogenicity of this parasite (Poulson and
317 Stensvold, 2016). The *Endolimax* sequences obtained in the present study exhibited ≥0.9%
318 dissimilarity to *E. nana*. Therefore, we are unable to confidently assign these sequences to the
319 species *E. nana*. These sequences may represent novel *Endolimax* species or genotypes, or other
320 *Endolimax* species that do not have 18S V9 sequences submitted to GenBank, which raises more
321 uncertainty about the pathogenic potential of *Endolimax* sp. obtained in our study. The close
322 relative of *Endolimax*, *Iodamoeba*, is also transmitted by faecal-oral contamination, and is an
323 intestinal parasite of humans, other primates, camels, pigs, rodents and birds (Stensvold et al.,

2012). Like *Endolimax*, *Iodamoeba* has generally been considered non-pathogenic, but some reports have found *Iodamoeba butschlii* infections in children and immunocompromised patients (Waywa et al., 2001; Faulkner et al., 2003). The *Iodamoeba* sequences obtained in this study were either 99.1% or 100% similar to *Iodamoeba* sp. isolated from human faeces (JN635741).

The percent sequence composition of *Blastocystis*, *Entamoeba*, *Endolimax*, *Iodamoeba* and *E. vermicularis* was overall higher in the influent and lower or not detected in the intermediate and effluent stages. It is possible that the wastewater treatment processes have reduced the faecal pathogen abundance compared to the influent. However, the number of sequences obtained by NGS does not represent the number of microorganisms present. A number of factors affect sequence composition, including PCR amplification bias (Hong et al., 2009), sequencing depth and copy number variation in the 18S gene. Quantitative assessments (e.g. counting parasite numbers) at different treatment stages are required to accurately determine the efficacy of pathogen removal during the water treatment process.

Other eukaryotic pathogens that were detected included the amoebozoans *Acanthamoeba* sp., which has a ubiquitous distribution in the environment and can cause blinding keratitis and fatal granulomatous encephalitis (Siddiqui and Khan, 2012), and *Vermamoeba vermiformis*, a free-living amoebae, which is a public health concern in drinking water, as it can harbor pathogenic bacteria or viruses and support the growth of bacteria including *Legionella pneumophila* (Delafont et al., 2018). Ubiquitous pathogenic green algae species (*Prototheca* sp.) that cause disease in humans and animals (Lass-Flörl and Mayr, 2007) were also found. Several oomycotic pathogens (fungi of the class Oomycetes) or genotypes similar to pathogenic species, such as *Lagenidium deciduum*, *Lagenidium* sp. and *Paralagenidium* sp. that cause mammalian infections (Spies et al., 2016), were also detected in the present study.

It is important for WWTPs to remove pathogens during the treatment process if the water is re-used for agricultural purposes (e.g. irrigation of food crops), as epidemiological studies

have shown that untreated wastewater used in agriculture is correlated with increased parasite infections in people (Amahmid et al., 1999). The removal of plant pathogens from wastewater re-used for crop irrigation is also important for the agricultural industry. *Pythium* sp. and *Phytopythium* sp. that cause crop disease were mainly found in the intermediate and effluent stages of WWTP 1, WWTP 2 and WWTP 4 (Supplementary File B.1).

Although the Euk1391F/EukBr primers from the Earth Microbiome Project's protocol were designed to amplify the eukaryotic 18S V9 region (www.earthmicrobiome.org/protocols-and-standards/18s/), the present study found that the primers cross-reacted with archaeal and bacterial 16S and were only slightly more biased towards the amplification of 18S (60% of total pre-processed reads). This may be due to the design of the forward primer (Euk1391F), which is very similar (only 2 bp downstream) to the universal (16S and 18S) primer 1389F designed by Amaral-Zettler et al. (2009). Our findings contrast with the study by Maritz et al. (2017), which used the same primers with NGS on sewage samples and reported "low numbers" of Archaea and Eubacteria amplification with Euk1391F/EukBr. In the present study, the mammalian blocking primer did work as expected, as only 0.03% of the processed sequences were mammalian 18S. Maritz et al. (2017) also compared the Euk1391F/EukBr (with and without the mammalian blocking primer) to the TAReuk454FWD1/TAReukREV3 eukaryotic 18S primers (Stoeck et al., 2009), which target the 18S V4 region, and reported that the TAReuk454FWD1/TAReukREV3 primers used for NGS resulted in sequences with higher variability and taxonomic accuracy (Maritz et al., 2017). Such results have also been shown by another study (Pawlowski et al., 2011). This suggests that the Euk1391F/EukBr primers are less suitable for taxonomic resolution, particularly due to the short sequence length (average length of 107 bp following adapter removal and primer trimming). The significant amount of 16S amplification that occurred in the current study reduced the sequencing depth of 18S (only 17/26 samples had adequate 18S depth, Figure 2), which is problematic for the detection of eukaryotic

374 pathogens that may be in low abundance in wastewater samples. Zoonotic protozoans such as
375 *Cryptosporidium*, *Giardia*, *Toxoplasma* and trichomonads were not detected with eukaryotic
376 18S NGS, but a more targeted *Cryptosporidium*-specific NGS approach was able to detect 12/26
377 *Cryptosporidium*-positive samples, and a total of nine different species were identified.

378 *Cryptosporidium* is an important human pathogen that can survive wastewater treatment
379 processes (Cheng et al., 2009), and 5/9 *Cryptosporidium* species identified in the present study
380 are zoonotic: *C. cuniculus*, *C. hominis*, *C. meleagridis*, *C. muris* and *C. parvum*. The most
381 prevalent species was *C. meleagridis*, which was detected in all four WWTPs. *Cryptosporidium*
382 *meleagridis* is the third most common *Cryptosporidium* species identified in humans in Australia
383 (Ng-Hublin et al., 2017) and is also a common parasite of birds including poultry (Zahedi et al.,
384 2016a). A recent study that examined the prevalence of *Cryptosporidium* in WWTPs across
385 three states in Australia also identified a high abundance of *C. meleagridis* in WA WWTPs, but
386 did not detect this species in New South Wales or Queensland WWTPs (Zahedi et al., 2018a).
387 The source of *C. meleagridis* in the present and the previous study is unknown. It is possible that
388 bird droppings could contaminate the intermediate stage of WWTP 1 maturation pond 1 as it is
389 open to birds, but *C. meleagridis* was also detected in the influent of WWTP 3. Since the
390 wastewater treated in these rural WWTPs comes primarily from household sewage systems,
391 humans appear to be a likely source of *C. meleagridis* in these WWTPs. The most common
392 *Cryptosporidium* species that infects humans, *C. hominis* and *C. parvum* (Zahedi et al., 2016a),
393 were also detected in all WWTPs, with the exception of WWTP 2. Of the two parasites, *C.*
394 *hominis* is by far the most dominant species in human populations in Australia and worldwide
395 (Ng-Hublin et al., 2017) and was detected in the influent in WWTP 4, but not in the effluent.
396 While cattle and Australian marsupials shed *C. hominis* oocysts (Hill et al., 2008; Ng et al.,
397 2011; Dowle et al., 2013; Vermeulen et al., 2015; Zahedi et al., 2016b; 2018b), the nature of the
398 sewerage system and WWTP locations result in humans being the likely source of *C. hominis* in

these WWTPs. *Cryptosporidium muris*, which was found in WWTP 1 and WWTP 4, is predominantly reported in rodents, but is also zoonotic (Wang et al., 2012), and is commonly identified in WWTPs (Huang et al., 2017; Zahedi et al., 2018a). *Cryptosporidium cuniculus* (only detected in WWTP 2 influent) infects rabbits, but also humans and was responsible for a waterborne outbreak of cryptosporidiosis in the UK (Puleston et al., 2014). *Cryptosporidium cuniculus* has also been reported in other WWTPs in Australia (Zahedi et al., 2018a). The non-zoonotic, cattle-derived *C. ryanae* was found in WWTP 4 effluent only, and interestingly, two *Cryptosporidium* piscine-derived genotypes (piscine genotype 4 and a potentially novel piscine genotype) were detected in WWTP 2 influent only. The source of the piscine-derived genotypes is unknown and unlikely to have come from fish-farms as WWTP 2 is inland and receives primarily household waste. Further research is required to determine if birds and rodents can also be reservoirs for piscine-genotypes.

Despite the lack of sensitivity for pathogen detection using the Euk1391F/EukBr primers with 18S NGS, an advantage of the method is that it allowed for other groups of eukaryotes to be identified, some of which are bioindicators of water treatment quality. The dominant groups of eukaryotes detected in the WSPs varied; for example, there was a high percent sequence composition of Chlorophyta (green algae) in the intermediate and effluent stages of WWTP 1 (53.2-61.1%), whereas the sequence composition of green algae was lower in the same treatment stages for WWTP 2 and WWTP 3 (2.3-25.6%). Chlorophyta are important components of WWTPs and remove nutrients including phosphorus and nitrogen and can improve the final effluent quality through “natural disinfection and incorporation of other contaminants, such as heavy metals, pharmaceuticals and endocrine disrupters” (Abinandan and Shanthakumar, 2015; Schulze et al., 2017). The reason for the lower number of Chlorophyta sequences in WWTP 2 and 3 is unknown but may have been affected by PCR amplification bias. The intermediate and effluent stages of the activated sludge plant (WWTP 4) had similar dominant groups of

eukaryotes as WSPs WWTP 2 and WWTP 3: Ciliophora (22.9-51.1%) and Euglenozoa (19.5-37.6%) (Figure 3). Previous studies on activated sludge plants have also identified many species of free-living protozoa, e.g. 160/228 protozoan species detected in activated sludge plants have been identified as Ciliophora (Curds, 1975). A drawback of the activated sludge treatment process is high sludge (bacteria) production, which is difficult to process with high nitrogen, phosphate and heavy metal concentrations (Ratsak et al., 1996). Protozoa that predate on bacteria are beneficial in activated sludge plants as this directly helps to reduce sludge. Well-functioning activated sludge plants have communities dominated by bacterivorous free-swimming ciliates (phylum Ciliophora) that swim in the liquor phase (Curds, 1966), crawling ciliates that move on the surface of sludge floc (e.g. the hypotrichs *Aspidisca* and *Euplotes*), and sessile ciliates that attach to the sludge floc (e.g. the peritrichs *Carchesium*, *Epistylis*, *Opercularia coarctata*, *Vorticella* and *Zoothamnium*) (Madoni, 2011). In the current study, within the Ciliophora, the subclass Peritrichia dominated, which is the most important group of bacterivorous ciliates in WWTPs and play a central role in effluent clarification, reducing biochemical oxygen demand (BOD) and turbidity (Madoni, 2003). The hypotrichs *Euplotes* sp. and the peritrichs *Epistylis* sp., and *Vorticella* sp. were detected in the intermediate and effluent stages for the activated sludge plant, WWTP 4, and were also detected in the WSPs (Supplementary File B.1). Other bacterivorous eukaryotes found were *Leptomyxa* sp., which is typically found in soil (Del Valle et al., 2017), the free-living nanoflagellates *Trimastix* sp. (Bernard et al., 2000), the aquatic fungi Cryptomycota, which parasitise phytoplankton such as diatoms, green algae, dinoflagellates and cyanobacteria (Rojas-Jimenez et al., 2017), and members of the phylum Loukozoa, which are free-living heterotrophs found in marine or freshwater environments (Simpson, 2016). The presence of so many bacterivorous eukaryotes indicates the lower technology WSPs, as well as the activated sludge plant, are functioning well, in a state where protozoa are predating on bacteria and reducing sludge quantities.

Several fungal communities were also found in the present study (Ascomycota, Basidiomycota, Blastocladiomycota, Cryptomycota, Chytridiomycota, Glomeromycota and Zygomycota). Ascomycota, Basidiomycota and Cryptomycota were among the most dominant groups and previous studies have also shown that these are the primary fungi in WWTPs (Weber et al., 2009; Evans and Seviour, 2012; Matsunaga et al., 2014). Members of the Ascomycota and Basidiomycota communities are capable of degrading cellulose, hemicellulose and lignin, and *Trichosporon* sp. (Basidiomycota) are involved in denitrification (Hayatsu et al., 2008; Matsunaga et al., 2014). Rotifers (a phylum of microscopic and near-microscopic pseudocoelomate animals), which had the highest percent sequence composition in WWTP 3 effluent (27.9%), are commonly found in WWTPs and play an important role in the removal of particulate matter (size range 0.2-10 μm) and also function as nuclei for floc formation in activated sludge (Lapinski and Tunnacliffe, 2003).

5. Conclusions

As NGS analysis of wastewater and particularly eukaryotic NGS analysis becomes more widely used, it will provide opportunities for the development of markers for pathogen source tracking, and a much better understanding of the importance of eukaryotic-mediated removal of pathogens and sludge quantities. However, it is also important to understand the limitations and biases of current assays. NGS analysis is not quantitative and quantification of the removal of pathogens throughout the treatment process is not possible. In order to provide a quantitative analysis, qPCR/ddPCR could be conducted on all microbes in the samples, however this would be expensive and much less taxonomically comprehensive. Another alternative would be to use NGS library preparation-methods that are PCR-free such as the Illumina TruSeq DNA Sample Prep kit, which are not subject to amplification bias, however this approach is also more expensive.

The present study has demonstrated that a eukaryotic 18S NGS approach with primers targeting the V9 region had inadequate sensitivity for detecting intestinal parasites in wastewater that are likely in low abundance. *Cryptosporidium*-specific NGS was required to detect nine species that were present in four WWTPs in WA. The eukaryotic 18S NGS approach did, however, detect *Blastocystis* sp. STs, *Endolimax* sp., *Entamoeba* sp., *E. vermicularis* and *Iodamoeba* sp., which have been associated with faecal-oral transmission routes that can occur via infected water supplies. While reuse options from these WWTPs do not include the possibility of infecting drinking water supplies, it is still important to achieve removal of parasitic species and human pathogens (e.g. *Cryptosporidium* sp.) in these WWTPs as WWTP 1, WWTP 2 and WWTP 4 reuse treated wastewater for irrigation of public open spaces. Removal of plant pathogens (e.g. *Pythium* sp., and *Phytopythium* sp.) that can cause crop disease is important if the treated wastewater is reused in agricultural applications. Several genotypes of the faecal pathogens identified in this study are potentially novel, including a potentially novel *Cryptosporidium* piscine genotype, and require further genetic characterisation for species and genotype confirmation in future studies. Investigations that aim to screen for faecal pathogens in wastewater should consider more targeted approaches, such as *Cryptosporidium*-specific NGS, for improved sensitivity.

The 18S NGS approach employed by this study also identified many eukaryotes that are important for the WSPs and activated sludge plants to function well, which included protozoa that predate on bacteria and reduce sludge quantities; Ascomycota and Basidiomycota communities that degrade cellulose, hemicellulose, and lignin; *Trichosporon* sp. (Basidiomycota) that is involved in denitrification; and Rotifers that play an important role in removal of particulate matter and in floc formation in activated sludge.

Appendices

Appendix A. Supplementary Data

Supplementary File A.1.

Acknowledgements

This work was supported by the Australian Research Council (ARC LP130100602), in collaboration with the Water Corporation of Western Australia and Water Research Australia. We thank Dr Elvina Lee, Annachiara Codello and Maninder Khurana for their assistance with next-generation sequencing preparation. We thank Arron Lethorn and operational staff at the Water Corporation of Western Australia for project collaboration, and assisting with site visits and sample collection.

Figure Legends

Figure 1. WWTP localities and different treatment stages sampled.

Figure 2. Alpha rarefaction plot of sequencing depth and ZOTUs detected. The plot was generated with the R package vegan (Oksanen et al., 2018) using R software (R Core Team, 2013).

Figure 3. Eukaryotic 18S NGS sequence percent composition plot of phyla detected in different treatment stages of wastewater sampled from WWTP 1-WWTP 4. Treatment stages include influent (I), intermediate (INT) and effluent (E). Phyla with ≤10% overall sequence composition are grouped as “other”. *Asterisk denotes that taxonomy was unassigned at the phylum level.

524 Figure 4. *Cryptosporidium* 18S NGS sequence percent composition plot for different treatment
525 stages of wastewater sampled from WWTP 1-WWTP 4. Treatment stages include influent (I),
526 intermediate (INT) and effluent (E).

References

Abinandan, S., Shanthakumar, S., 2015. Challenges and opportunities in application of microalgae (Chlorophyta) for wastewater treatment: A review. Renew. Sustain. Energy Rev. 52, 123-132.

Alfellani, M.A., Taner-Mulla, D., Jacob, A.S., Imeede, C.A., Yoshikawa, H., Stensvold, C.R., Clark, C.G., 2013. Genetic diversity of *Blastocystis* in livestock and zoo animals. Protist. 164, 497-509.

Amahmid, O., Asmama, S., Bouhoum, K., 1999. The effect of waste water reuse in irrigation on the contamination level of food crops by *Giardia* cysts and *Ascaris* eggs. Int. J. Food Microbiol. 49, 19-26.

Amaral-Zettler, L.A., McCliment, E.A., Ducklow, H.W., Huse, S.M., 2009. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. PLoS ONE. 4(7), e6372.

Amoah, I.D., Adegoke A.A., Stenström T.A., 2018. Soil-transmitted helminth infections associated with wastewater and sludge reuse: A review of current evidence. Trop. Med. Int. Health. 23, 692-703.

Azizian, M., Basati, G., Abangah, G., Mahmoudi, M.R., Mirzaei, A., 2016. Contribution of *Blastocystis hominis* subtypes and associated inflammatory factors in development of irritable bowel syndrome. Parasitol. Res. 115, 2003-2009.

Ben Ayed, L., Schijven, J., Alouini, Z., Jemli, M., Sabbahi, S., 2009. Presence of parasitic protozoa and helminths in sewage and efficacy of sewage treatment in Tunisia. Parasitol. Res. 105, 393-406.

550 Berglund, B., Dienus, O., Sokolova, E., Berglind, E., Matussek, A., Pettersson, T.,
551 Lindgren, P.E., 2017. Occurrence and removal efficiency of parasitic protozoa in
552 Swedish wastewater treatment plants. Sci. Total Environ. 598, 821-827.
553 Bernard, C., Simpson, A.G.B., Patterson, D.J., 2000. Some free-living flagellates
554 (Protista) from anoxic habitats. Ophelia. 52, 113-142.
555 Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley,
556 G.A., Caporaso, J.G., 2018. Optimizing taxonomic classification of marker-gene
557 amplicon sequences with qiime 2's q2-feature-classifier plugin. Microbiome. 6,
558 90.
559 Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello,
560 E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley,
561 S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D.,
562 Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters,
563 W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight. R., 2010. QIIME
564 allows analysis of high-throughput community sequencing data. Nat. Methods. 7,
565 335-336.
566 Cheng. H.W., Lucy. F., Graczyk, T.K., Broaders, M.A., Tamang, L., Connolly, M., 2009.
567 Fate of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and
568 *Giardia duodenalis* cysts during secondary waste-water treatments. Parasitol. Res.
569 105, 689-696.
570 Cifuentes, E., Blumental, U., Ruiz-Palacios, G., Bennett, S., Peasey, A., 1994.
571 Epidemiologic setting of the agricultural use of sewage. Valle del Mezquital,
572 Mexico. Salud. Publica. de Mexico. 36, 3-9.

573 Clark, C.G., Diamond, L.S., 1991. Ribosomal RNA genes of 'pathogenic' and
574 'nonpathogenic' *Entamoeba histolytica* are distinct. Mol. Biochem. Parasitol. 49,
575 297-302.
576 Curds, C.R., 1966. An ecological study of the ciliated protozoa in activated-sludge
577 process. Oikos. 15, 282-289.
578 Curds, C.R., 1975. Protozoa. In: Ecological aspects of used-water treatment. Edited by:
579 Curds, C.R., Hawkes, H.A., London: Academic Press. pp. 203-268.
580 Delafont, V., Rodier, M.H., Maisonneuve, E., Cateau, E., 2018. *Vermamoeba*
581 *vermiformis*: a free-living amoeba of interest. Microb Ecol. 76(4), 991-1001.
582 Del Valle, A.D.O.F., Lorenzo-Morales, J., Maciver, S.K., 2017. *Leptomyxa valladaresi* n.
583 sp. (Amoebozoa, Tubulinea, Leptomyxida), from Mount Teide, Tenerife, Spain.
584 Exp. Parasitol. 183, 85-91.
585 Dowle, M., Hill, N.J., Power, M.L., 2013. *Cryptosporidium* from free ranging marsupial
586 host: Bandicoots in urban Australia. Vet. Parasitol. 198, 197-200.
587 Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST.
588 Bioinformatics. 26, 2460-2461.
589 Edgar, R.C., 2016. UNOISE2: improved error-correction for Illumina 16S and ITS
590 amplicon sequencing. bioRxiv 081257. doi: https://doi.org/10.1101/081257.
591 Eland, L.E., Davenport, R.J., Santos, A.B., Mota Filho, C.R., 2018. Molecular evaluation
592 of microalgal communities in full-scale waste stabilisation ponds. Environ.
593 Technol. In Press. 22, 1-8.
594 Evans, T.N., Seviour, R.J., 2012. Estimating biodiversity of fungi in activated sludge
595 communities using culture-independent methods. Microb. Ecol. 63, 773-786.

596 Faulkner, C.T., Garcia, B.B., Logan, M.H., New, J.C., Patton, S., 2003. Prevalence of
597 endoparasitic infection in children and its relation with cholera prevention efforts
598 in Mexico. Rev. Panam. Salud. Publica. 14, 31-41.
599 Fonseca, J.A., Heredia, R.D., Ortiz, C., Mazo, M., Clavijo-Ramírez, C.A., Lopez, M.C.,
600 2016. Identification of *Entamoeba moshkovskii* in treated waste water used for
601 agriculture. Ecohealth. 13, 156-60.
602 Forsell, J., Granlund, M., Stensvold, C.R., Clark, C.G., Evengård, B., 2012. Subtype
603 analysis of *Blastocystis* isolates in Swedish patients. Eur. J. Clin. Microbiol.
604 Infect. Dis. 31, 1689-1696.
605 Fletcher, S.M., Stark, D., Harkness, J., Ellis, J., 2012. Enteric protozoa in the developed
606 world: a public health perspective. Clin. Microbiol. Rev. 25, 420-449.
607 Fotedar, R., Stark, D., Beebe, N., Marriott, D., Ellis, J. and Harkness, J., 2007. Laboratory
608 diagnostic techniques for *Entamoeba* species. Clin. Microbiol. Rev. 20, 511-532.
609 Garrido-Cardenas, J.A., Polo-López, M.I., Oller-Alberola, I., 2017. Advanced microbial
610 analysis for wastewater quality monitoring: metagenomics trend. Appl. Microbiol.
611 Biotechnol. 101, 7445-7458.
612 Graczyk, T.K., Shiff, C.K., Tamang, L., Munsaka, F., Beitin, A.M., Moss, W.J., 2005.
613 The association of *Blastocystis hominis* and *Endolimax nana* with diarrheal stools
614 in Zambian school-age children. Parasitol. Res. 98, 38-43.
615 Hayatsu, M., Tago, K., Saito, M., 2008. Various players in the nitrogen cycle: diversity
616 and functions of the microorganisms involved in nitrification and denitrification.
617 Soil Sci. Plant Nutr. 54, 33-45.
618 Hill, N.J., Deane, E.M., Power M.L., 2008. Prevalence and genetic characterization of
619 *Cryptosporidium* isolates from common brushtail possums (*Trichosuris*
620 *vulpecula*) adapted to urban settings. Appl. Environ. Microbiol. 74, 5549-5555.

621 Hong, S., Bunge, J., Leslin, C., Jeon, S., Epstein, S.S., 2009. Polymerase chain reaction
622 primers miss half of rRNA microbial diversity. ISME J. 3, 1365-1373.
623 Huang, C., Hu, Y., Wang, L., Wang, Y., Li, N., Guo, Y., Feng, Y., Xiao, L., 2017.
624 Environmental transport of emerging human-pathogenic *Cryptosporidium* species
625 and subtypes through combined sewer overflow and wastewater. Appl. Environ.
626 Microbiol. 83, e00682-17.
627 Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S.,
628 Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P.,
629 Drummond, A., 2012. Geneious Basic: An integrated and extendable desktop
630 software platform for the organization and analysis of sequence data.
631 Bioinformatics. 28, 1647-1649.
632 King, B., Fanok, S., Phillips, R., Lau, 670 M., van den Akker, B., Monis, P., 2017.
633 *Cryptosporidium* attenuation across the wastewater treatment train: Recycled
634 water fit for purpose. Appl. Environ. Microbiol. 83, e03068-16.
635 Lapinski, J., Tunnacliffe, A., 2003. Reduction of suspended biomass in municipal
636 wastewater using bdelloid rotifers. Water Res. 37, 2027-2034.
637 Lass-Flörl, C., Mayr, A., 2007. Human protothecosis. Clin. Microbiol. Rev. 20, 230-242.
638 Levecke, B., Dorny, P., Vercammen, F., Visser, L.G., Van Esbroeck, M., Vercruysse, J.,
639 Verweij, J.J., 2015. Transmission of *Entamoeba nuttalli* and *Trichuris trichiura*
640 from nonhuman primates to humans. Emerg. Infect. Dis. 21, 1871-1872.
641 Madoni, P., 2003. Protozoa as indicators of wastewater treatment efficiency. In: Mara, D
642 and Horan, N Eds), Handbook of Water and Wastewater Microbiology. Elsevier.
643 pp 361-371. ISBN: 978-0-12-470100-7.
644 Madoni, P., 2011. Protozoa in wastewater treatment processes: A minireview. Ital. J.
645 Zool. 78, 3-11.

646 Maritz, J.M., Rogers, K.H., Rock, T.M., Liu, N., Joseph, S., Land, K.M., Carlton, J.M.,
647 2017. An 18S rRNA workflow for characterizing protists in sewage, with a focus
648 on zoonotic trichomonads. Microb. Ecol. 74, 923-936.
649 Matsunaga, K., Kubota, K., Harada, H., 2014. Molecular diversity of eukaryotes in
650 municipal wastewater treatment processes as revealed by 18S rRNA gene
651 analysis. Microbes Environ. 29, 401-407.
652 Morgan, U.M., Constantine, C.C., Forbes, D.A., Thompson, R.C., 1997. Differentiation
653 between human and animal isolates of *Cryptosporidium parvum* using rDNA
654 sequencing and direct PCR analysis. J. Parasitol. 83(5), 825-830.
655 Nasser, A.M., Vaizel-Ohayon, D., Aharoni, A., Revhun, M., 2012. Prevalence and fate of
656 *Giardia* cysts in wastewater treatment plants. J. Appl. Microbiol. 113, 477-484.
657 Ng, J., Yang, R., Whiffin, V., Cox, P., Ryan, U., 2011. Identification of zoonotic
658 *Cryptosporidium* and *Giardia* genotypes infecting animals in Sydney's water
659 catchments. Exp. Parasitol. 128, 138-144.
660 Ng-Hublin, J.S.Y., Combs, B., Reid, S., Ryan, U., 2017. Differences in the occurrence
661 and epidemiology of cryptosporidiosis in Aboriginal and non-Aboriginal people in
662 Western Australia (2002-2012). Infect. Genet. Evol. 53, 100-106.
663 Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin,
664 P.R., O'Hara, R. B., Simpson, G.L., Solymos, P., Henry, M., Stevens, H., Szoecs,
665 E., Wagner, H., 2018. Vegan: Community ecology package. R package version
666 2.5-2. https://CRAN.R-project.org/package=vegan.
667 Paparini, A., Gofton, A., Yang, R., White, N., Bunce, M., Ryan, U.M., 2015. Comparison
668 of Sanger and next generation sequencing performance for genotyping
669 *Cryptosporidium* isolates at the 18S rRNA and actin loci. Exp. Parasitol. 151-152,
670 21-27.

671 Pawlowski, J., Christen, R., Lecroq, B., Bachar, D., Shahbazkia, H.R., Amaral-Zettler, L.,
672 Guillou, L., 2011. Eukaryotic richness in the abyss: insights from pyrotag
673 sequencing. PLoS One. 6, e18169.
674 Poulsen, C.S., Stensvold, C.R., 2016. Systematic review on Endolimax nana: A less well
675 studied intestinal ameba. Trop. Parasitol. 6, 8-29.
676 Puleston, R.L., Mallaghan, C.M., Modha, D.E., Hunter, P.R., Nguyen-Van-Tam, J.S.,
677 Regan, C.M., Nichols, G.L., Chalmers, R.M., 2014. The first recorded outbreak of
678 cryptosporidiosis due to *Cryptosporidium cuniculus* (formerly rabbit genotype),
679 following a water quality incident. J. Water Health. 12, 41-50.
680 Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J.,
681 Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project:
682 improved data processing and web-based tools. Nucl. Acids Res. 41, D590-D596.
683 R Core Team, 2013. R: A language and environment for statistical computing. R
684 foundation for statistical computing, Vienna, Austria. URL http://www.R-
685 project.org/.
686 Rajasulochana, R., Preethy, V., 2016. Comparison on efficiency of various techniques in
687 treatment of waste and sewage water - A comprehensive review. Resource-
688 Efficient Technologies, 2, 175-184.
689 Ramírez, J.D., Sánchez, A., Hernández, C., Flórez, C., Bernal, M.C., Giraldo, J.C., Reyes,
690 P., López, M.C., García, L., Cooper, P.J., Vicuña, Y., Mongi, F., Casero, R.D.,
691 2016. Geographic distribution of human *Blastocystis* subtypes in South America.
692 Infect. Genet. Evol. 41, 32-35.
693 Ratsak, C.H., Maarsen, K.A., Kooijman, S.A.L.M., 1996. Effects of protozoa on carbon
694 mineralization in activated sludge. Water Res. 30, 1-12.

695 Rojas-Jimenez, K., Wurzbacher, C., Bourne, E.C., Chiuchiolo, A., Priscu, J.C. Grossart,
696 H.P., 2017. Early diverging lineages within *Cryptomycota* and *Chytridiomycota*
697 dominate the fungal communities in ice-covered lakes of the McMurdo Dry
698 Valleys, Antarctica. Scientific reports. 7, 15348.
699 Schulze, P. S. C., Carvalho, C. F. M., Pereira, H., Gangadhar, K. N., Schüler, L. M.,
700 Santos, T. F., Varela, J. C. S., Barreira, L., 2017. Urban wastewater treatment by
701 Tetraselmis sp. CTP4 (Chlorophyta). Bioresour. Technol. 223, 175-183.
702 Shah, M., Tan, C.B., Rajan, D., Ahmed, S., Subramani, K., Rizvon, K., Mustacchia, P.,
703 2012. *Blastocystis hominis* and *Endolimax nana* co-infection resulting in chronic
704 diarrhea in an immunocompetent male. Case Rep. Gastroenterol. 6, 358-364.
705 Shimokawa, C., Kabir, M., Taniuchi, M., Mondal, D., Kobayashi, S., Ali, I.K., Sobuz,
706 S.U., Senba, M., Houpt, E., Haque, R., Petri, W.A., Hamano, S., 2012. *Entamoeba*
707 *moshkovskii* is associated with diarrhea in infants and causes diarrhea and colitis
708 in mice. J. Infect. Dis. 206, 744-751.
709 Siddiqui, R., Khan, N.A., 2012. Biology and pathogenesis of *Acanthamoeba*. Parasit.
710 Vectors. 5, 6.
711 Simpson, A.G.B., 2016. Jakobida. In: Archibald, J., Simpson, M., Alastair, G.B.,
712 Slamovits, C.H., (eds) Handbook of the Protists. Springer, Cham.
713 Spies, C.F., Grooters, A.M., Lévesque, C.A., Rintoul, T.L., Redhead, S.A., Glockling,
714 S.L., Chen, C.Y., De Cock, A.W., 2016. Molecular phylogeny and taxonomy of
715 *Lagenidium*-like oomycetes pathogenic to mammals. Fungal Biol. 120, 931-947.
716 Stensvold, C.R., Suresh, G.K., Tan, K.S., Thompson, R.A., Traub, R.J., Viscogliosi, E.,
717 Yoshikawa, H., Clark, C.G., 2007. Terminology for *Blastocystis* subtypes-a
718 consensus. Trends Parasitol. 23, 93-96.

719 Stensvold, C.R., Lebbad, M., Clark, C.G., 2012. Last of the human protists: the
720 phylogeny and genetic diversity of *Iodamoeba*. Mol. Biol. Evol. 29, 39-42.
721 Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M.D., Breiner, H.W., Richards, T.A.,
722 2010. Multiple marker parallel tag environmental DNA sequencing reveals a
723 highly complex eukaryotic community in marine anoxic water. Mol. Ecol. 19
724 (Suppl 1), 21-31.
725 Toro Monjaraz, E.M., Vichido Luna, M.A., Montijo Barrios, E., Cervantes Bustamante,
726 R., Zárate Mondragón, F., Huante Anaya, A., Cadena León, J., Mendez, M.C.,
727 López Ugalde, M., Ramirez Mayans, J.A., 2017. *Blastocystis hominis* and chronic
728 abdominal pain in children: Is there an association between them? J. Trop. Pediatr.
729 64, 279-283.
730 Vermeulen, E.T., Ashworth, D.L., Eldridge, M.D.B., Power, M.L., 2015. Diversity of
731 *Cryptosporidium* in brush tailed rock wallabies (*Petrogale penicillata*) managed
732 within a species recovery programme. Int. J. Parasitol. Parasite. Wildl. 4, 190-196.
733 Wang, R., Jian, F., Zhang, L., Ning, C., Liu, A., Zhao, J., Feng, Y., Qi, M., Wang, H., Lv,
734 C., Zhao, G., Xiao, L., 2012. Multilocus sequence subtyping and genetic structure
735 of *Cryptosporidium muris* and *Cryptosporidium andersoni*. PLoS One. 7, e43782.
736 Weber, S.D., Hofmann, A., Pilhofer, M., Wanner, G., Agerer, R., Ludwig, W., Schleifer,
737 K.H., Fried, J., 2009. The diversity of fungi in aerobic sewage granules assessed
738 by 18S rRNA gene and ITS sequence analyses. FEMS Microbiol. Ecol. 68, 246-
739 254.
740 Waywa, D., Kongkriengdaj, S., Chaidatch, S., Tiengrim, S., Kowadisaiburana, B.,
741 Chaikachonpat, S., Suwanagool, S., Chaiprasert, A., Curry, A., Bailey, W.,
742 Suputtamongkol, Y., Beeching, N.J., 2001. Protozoan enteric infection in AIDS

related diarrhea in Thailand. Southeast Asian J. Trop. Med. Public Health. 32, 151-155.

Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A.A., Montali, R.J., Fayer, R., Lal, A.A., 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl. Environ. Microbiol. 65, 1578-1583.

Xu, S., Yao, J., Ainiwaer, M., Hong, Y., Zhang, Y., 2018. Analysis of bacterial community structure of activated sludge from wastewater treatment plants in winter. Biomed. Res. Int. 2018, 8278970.

Yang, R., Murphy, C., Song, Y., Ng-Hublin, J., Estcourt, A., Hijjawi, N., Chalmers, R., Hadfield, S., Bath, A., Gordon, C., Ryan, U., 2013. Specific and quantitative detection and identification of *Cryptosporidium hominis* and *C. parvum* in clinical and environmental samples. Exp. Parasitol. 135, 142-147.

Yang, R., Jacobson, C., Gardner, G., Carmichael, I., Campbell, A.J., Ryan, U., 2014. Development of quantitative PCR (qPCR) for *Giardia* and analysis of prevalence, cyst shedding and genotypes of *Giardia* present in sheep across four states in Australia. Exp. Parasitol. 137, 46-52.

Zahedi, A., Paparini, A., Jian, F., Robertson, I., Ryan, U., 2016a. Public health significance of zoonotic *Cryptosporidium* species in wildlife: critical insights into better drinking water management. Int. J. Parasitol: Parasit. Wildl. 5, 88-109.

Zahedi, A., Monis, P., Aucote, S., King, B., Paparini, A., Jian, F., Yang, R., Oskam, C., Ball, A., Robertson, I., Ryan, U., 2016b. Zoonotic *Cryptosporidium* species in animals inhabiting Sydney water catchments. PLoS One. 11, e0168169.

Zahedi, A., Gofton, A.W., Greay, T., Monis, P., Oskam, C., Ball, A., Bath, A., Watkinson, A., Robertson, I., Ryan, U., 2018a. Profiling the diversity of

Cryptosporidium species and genotypes in wastewater treatment plants in Australia using next generation sequencing. Sci. Total Environ. 644, 635-648.

Zahedi, A., Monis, P., Gofton, A.W., Oskam, C.L., Ball, A., Bath, A., Bartkow, M. Robertson, I., Ryan, U., 2018b. *Cryptosporidium* species and subtypes in animals inhabiting drinking water catchments in three states across Australia. Water Res. 134, 327-340.

Zaman, V., Khan, K.Z., Khan, M.A., Khan, M.A., 1994. Isolation of *Blastocystis hominis* from sewage. Southeast Asian J. Trop. Med. Public Health. 25, 211.

Table 5. 18S sequence compositions (%) of eukaryotic bioindicators in WWTP influent (I), intermediate (INT) and effluent (E) samples

Eukaryotic bioindicators	Phylum	WWTP 1			WWTP 2		WWTP 3			WWTP 4		
		I	INT	E	I	E	I	INT	E	I	INT	E
Bacterivorous eukaryotes	Ciliophora	0.6	0.1	8.8	11.7	39.4	2.1	0.1	22.9	1.4	35.2	51.1
	Loukozoa	-	0.2	0.1	<0.1	<0.1	-	-	<0.1	-	0.1	0.2
Fungal communities	Ascomycota	15.8	<0.1	0.1	<0.1	<0.1	12.8	8.1	<0.1	9.1	0.2	0.1
	Basidiomycota	0.8	0.1	0.1	-	<0.1	2.4	0.6	<0.1	0.6	<0.1	0.1
	Blastocladiomycota	<0.1	<0.1	<0.1	-	-	-	-	<0.1	-	<0.1	<0.1
	Chytridiomycota	<0.1	<0.1	1.1	-	<0.1	1.5	-	-	0.2	<0.1	<0.1
	Cryptomycota	14.5	<0.1	0.2	<0.1	<0.1	9.5	<0.1	-	4.0	5.6	0.8
	Glomeromycota	<0.1	<0.1	<0.1	-	-	0.2	-	-	0.1	<0.1	<0.1
	Hyphochytriomycota	<0.1	-	<0.1	-	-	0.1	-	-	<0.1	5.5	0.6
	Zygomycota	0.6	<0.1	1.1	-	<0.1	-	-	-	<0.1	-	-
Green algae	Chlorophyta	3.5	61.1	53.2	16.1	3.6	2.3	25.6	2.3	48.1	0.2	0.5
Rotifers	Rotifera	1.7	0.5	9.5	0.5	2.1	2.4	0.2	27.9	1.0	0.3	1.6
Other	Other	62.5	38.1	25.8	71.7	54.9	66.8	65.4	46.9	35.6	52.9	45.0

Table 1. Rural wastewater treatment plant samples analysed in the present study

WWTP	Treatment technology	Location	Climate	Sample ID	Wastewater treatment stage	Sample collection date; season
WWTP 1	Stabilisation pond: Combined anaerobic and aerobic pond system, followed by two maturation ponds	Northwest Western Australia	Tropical climate. Wet and dry seasons.	WWTP 1-1	Influent	19-Feb-2015; Wet
				WWTP 1-2	Effluent (pre-chlorination)	
				WWTP 1-3	Effluent (post-chlorination)	
				WWTP 1-4	Influent	7-Sep-2015; Dry
				WWTP 1-5	Intermediate (post maturation pond 1)	
				WWTP 1-6	Intermediate (post maturation pond 2)	
				WWTP 1-7	Effluent (pre-chlorination)	
				WWTP 1-8	Effluent (post-chlorination)	
WWTP 2	Stabilisation pond: One facultative pond	Wheatbelt, Western Australia	Hot dry summers and mild winters. Four distinct seasons.	WWTP 2-1	Influent	12-Feb-2015; Summer
				WWTP 2-2	Effluent (final pond)	
				WWTP 2-3	Effluent (storage basin)	
				WWTP 2-4	Influent	13-Jul-2015; Winter
				WWTP 2-5	Effluent (final pond)	
				WWTP 2-6	Effluent (storage basin)	

WWTP 3	Stabilisation pond: Two primary facultative ponds, and one secondary pond	Southwest Western Australia	Temperate climate. Four distinct seasons	WWTP 3-1	Influent	23-Feb-2015; Summer
				WWTP 3-2	Intermediate (post-pond)	
				WWTP 3-3	Effluent	
				WWTP 3-4	Influent	14-July-2015; Winter
				WWTP 3-5	Intermediate (post-pond)	
				WWTP 3-6	Effluent	
WWTP 4	Activated sludge: Oxidation ditches followed by sedimentation tanks	Southwest Western Australia	Temperate climate. Four distinct seasons	WWTP 4-1	Influent	23-Feb-2015; Summer
				WWTP 4-2	Intermediate (oxidation ditch)	
				WWTP 4-3	Effluent	
				WWTP 4-4	Influent	14-July-2015; Winter
				WWTP 4-5	Intermediate (oxidation ditch)	
				WWTP 4-6	Effluent	

Table 2. Details of 18S primers used for NGS.

Target organisms	Primer names	Primer sequences (5'-3')	Product size (bp)	Annealing Temperature (°C)	References
Cryptosporidium sp.	18S iF	AGTGACAAGAAATAACAATACAGG	~298	60	Morgan et al., 1997
	18S iR	CCTGCTTTAAGCACTCTAATTTTC			
Eukaryotes	Euk1391F	GTACACACCGCCCGTC	~107[a]	65	www.earthmicrobiome.org/protocols-and-standards/18s/
	EukBr	TGATCCTTCTGCAGGTTCACCTAC			
Mammals	Mammalian blocking primer Mammal_block_I-short_1391f	GCCCGTCGCTACTACCGATTGG44444TTAGTGAGGCC3[b]	-		

[a]Average sequence length after adapter removal and primer trimming
[b]3 = C3 spacer; 4 = deoxyinosine

Table 3. Eukaryotic and *Cryptosporidium*-specific 18S NGS sequence statistics

Eukaryotic 18S NGS sequences						
	Raw (unprocessed)	**Pre-processed**[a]	**Processed 18S sequences**[b]			
Statistics	**Grand total (*n* = 36)**		**Samples (*n* = 26)**	**Extraction controls (*n* = 6)**	**NTCs (*n* = 4)**	**Grand total (*n* = 36)**
Average	88,647	68,783	56,394	3,044	1,776	41,434
Standard deviation	98,350	75,226	60,064	4,573	1,419	56,380
Min	47	34	587	9	353	9
Max	399,120	353,900	269,925	12,243	3,230	269,925
Total	4,432,328	2,476,189	1,466,243	18,265	7,104	1,491,612

***Cryptosporidium*-specific 18S NGS sequences**					
	Raw	**Pre-processed**[a]	**Processed *Cryptosporidium* 18S sequences**[c]		
Statistics	**Grand total (*n* = 33)**		**Samples (12/26)**[d]	**Extraction controls (*n* = 6)**	**NTCs (*n* = 1)**
Average	13,247	6,170	1,649	-	-
Standard deviation	13,230	3,315	2,088	-	-
Min	970	715	11	0	0
Max	49,932	12,254	5,464	0	0
Total	304,692	141,916	19,790	0	0

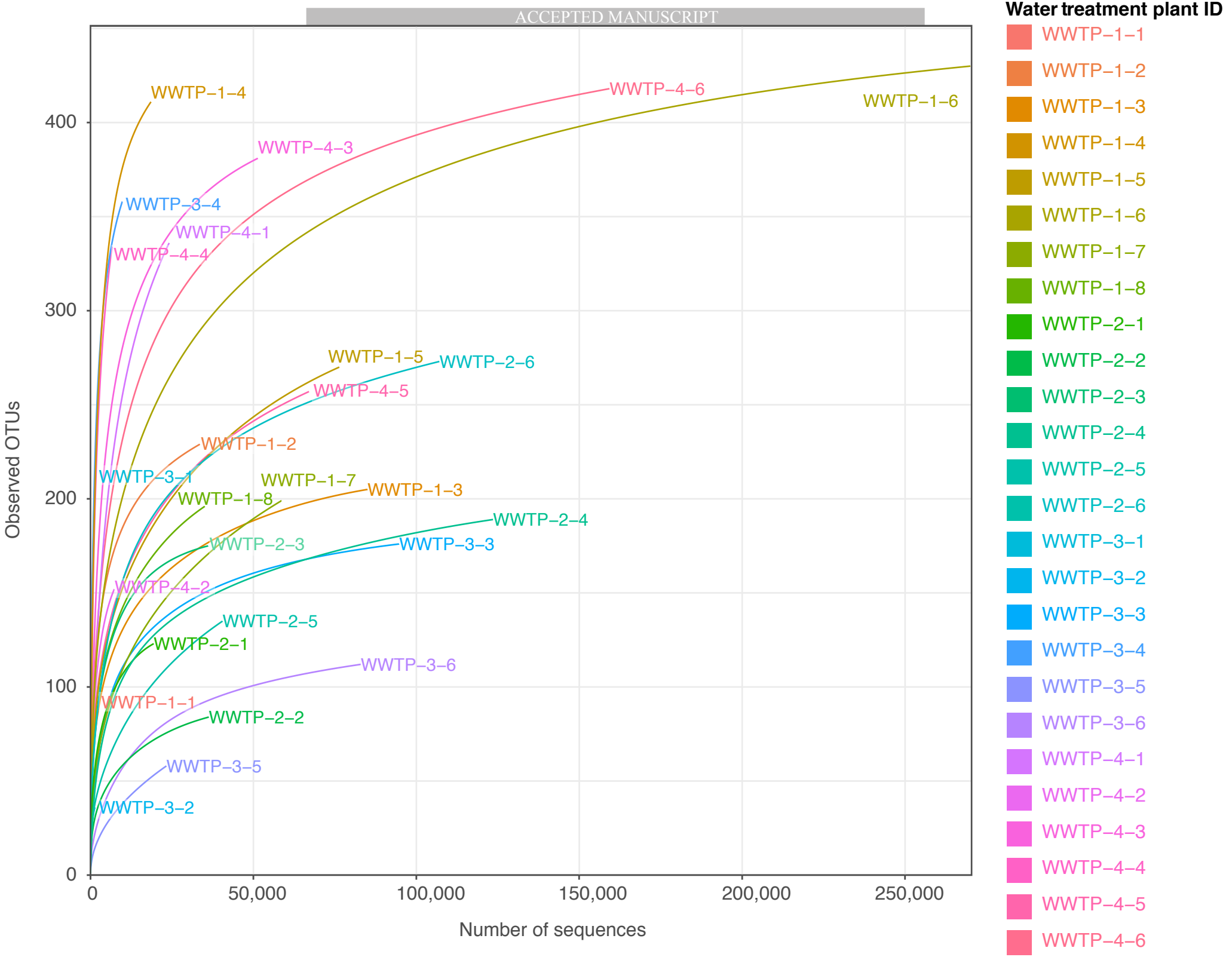
[a]Merged, quality filtered sequences with singletons, chimeras and contaminant ZOTUs removed
[b]Merged, quality filtered sequences with singletons, chimeras, contaminant ZOTUs, bacterial and archaeal 16S sequences removed
[c]Merged, quality filtered sequences with singletons, contaminant ZOTUs and non-*Cryptosporidium* sequences removed
[d]Number of *Cryptosporidium*-positive samples over total sample number

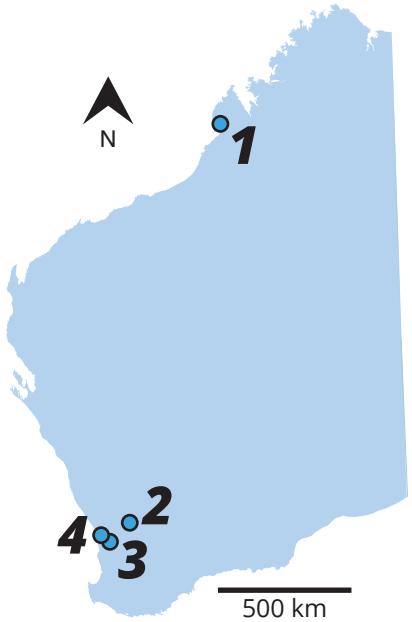
Table 4. Intestinal parasite sequence compositions (%) obtained with eukaryotic 18S NGS in WWTP influent (I), intermediate (INT) and effluent (E) samples

						WWTP 1			WWTP 2		WWTP 3			WWTP 4		
Phylum	**ZOTU No.**	**Accession number**	**Species**	**Top BLAST hit accession**	**Similarity (%)**	**I**	**INT**	**E**	**I**	**E**	**I**	**INT**	**E**	**I**	**INT**	**E**
Amoebozoa	1158; 1920; 2011; 2022; 2159; 2430; 2670; 2799; 2875; 3104; 3185; 3214	MH623054; MH623058; MH623059; MH623060; MH623062; MH623064; MH623065; MH623067; MH623068; MH623070; MH623071; MH623072	*Endolimax* spp.	AF149916	94.0-99.1	0.7	<0.1	-	-	-	0.6	<0.1	-	0.1	-	<0.1
	485	MH623050	*Entamoeba coli*	AF149915	100	2.2	-	-	-	-	0.7	-	-	0.2	<0.1	<0.1
	1563	MH623056	*Entamoeba hartmanni*	KX618191		<0.1	-	-	-	-	0.2	-	-	0.1	-	-
	2095	MH623061	*Entamoeba moshkovskii*	KP722601		<0.1	-	-	-	-	0.1	-	-	<0.1	<0.1	-
	538	MH623051	*Entamoeba polecki*	LC082304		0.8	-	<0.1	-	-	-	-	-	-	-	-
	1622	MH623057	*Entamoeba* sp. ZOTU 1622[a]	KP722601	93.5	-	-	-	-	-	<0.1	-	-	0.2	<0.1	-
	2260	MH623063	*Entamoeba* sp. ZOTU 2260[b]		91.7	<0.1	-	-	-	-	<0.1	-	-	<0.1	<0.1	-
	405	MH623049	*Entamoeba* sp. ZOTU 405[c]		96.2	<0.1	-	-	-	-	0.4	-	-	2.9	<0.1	-
	832	MH623052	*Entamoeba* sp. ZOTU 832[d]	LC041205	100	0.4	-	<0.1	-	-	0.4	-	-	0.1	<0.1	<0.1
	2906	MH623069	*Iodamoeba* sp. ZOTU 2906	JN635741	100	<0.1	-	-	-	-	-	-	-	<0.1	-	-
	3246	MH623073	*Iodamoeba* sp. ZOTU 3246	JN635741	99.1	0.1	-	-	-	-	-	-	-	-	-	-
Nematoda	327	MH623047	*Enterobius vermicularis*	JF934731	100	1.8	-	-	-	-	-	-	-	-	-	-
Sarcomastigophora	124	MH623043	*Blastocystis* sp. ST1	KM213500	100	6.4	<0.1	<0.1	-	<0.1	7.9	0.1	-	2.6	-	<0.1
	244	MH623045	*Blastocystis* sp. ST2	KM213503		2.3	<0.1	<0.1	<0.1	<0.1	4.9	<0.1	<0.1	0.8	<0.1	<0.1
	203	MH623044	*Blastocystis* sp. ST3	KX618192		3.6	<0.1	<0.1	<0.1	<0.1	1.7	-	-	1.3	<0.1	<0.1

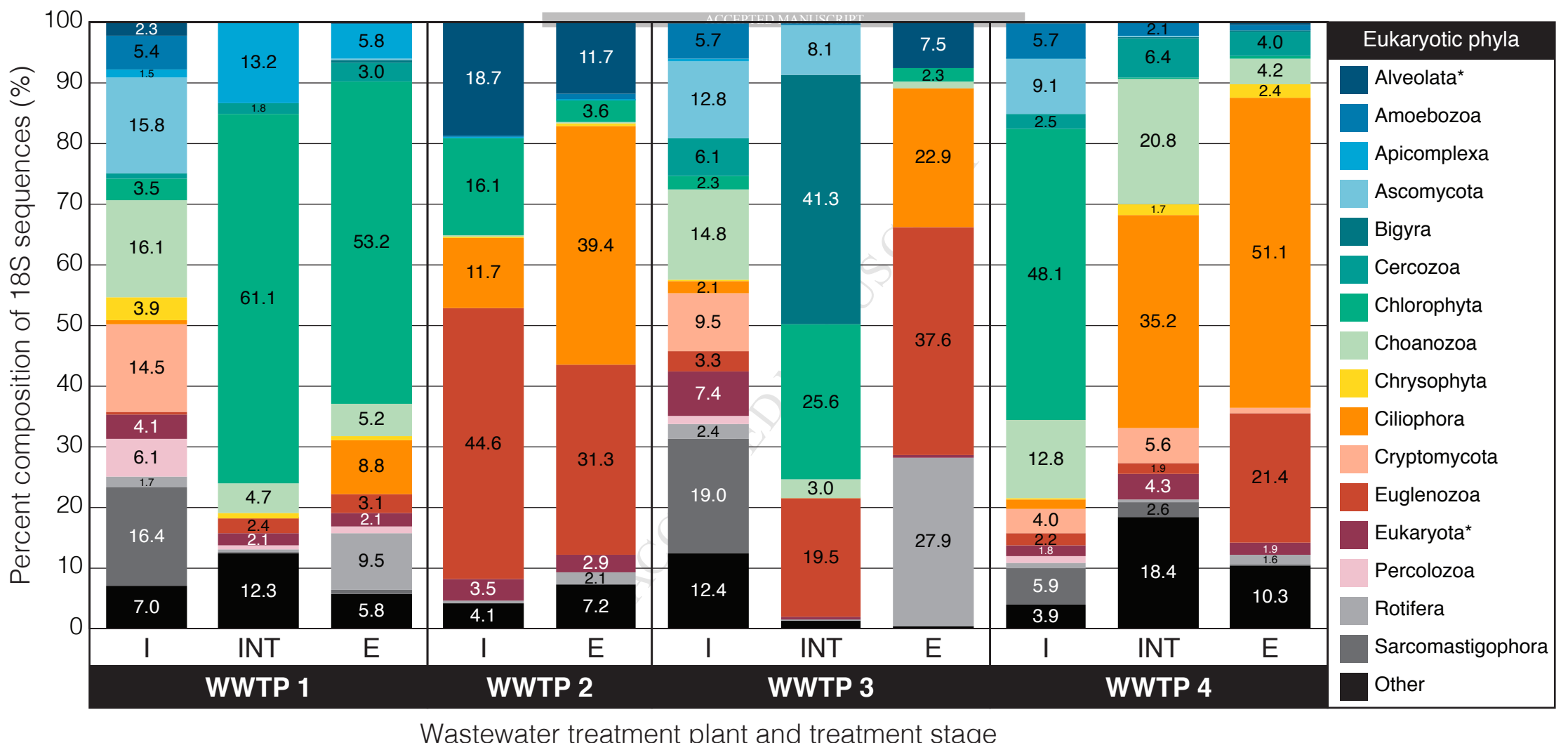
	288	MH623046	*Blastocystis* sp. ST4	KU939393		2.4	<0.1	<0.1	<0.1	<0.1	2.7	<0.1	-	1.0	<0.1	<0.1
	2740	MH623066	*Blastocystis* sp. ST6	EU445485		0.2	-	-	-	-	<0.1	-	-	<0.1	-	-
	345	MH623048	*Blastocystis* sp. ST8	AB107971		1.4	<0.1	<0.1	-	-	0.7	-	-	0.2	<0.1	<0.1
	1064	MH623053	*Blastocystis* sp. ZOTU 1064[e]	JN682513	94.4	<0.1	-	-	-	-	0.6	-	-	<0.1	-	-
	1314	MH623055	*Blastocystis* sp. ZOTU 1314[f]	KU939393	96.8	-	-	-	-	-	0.4	-	-	<0.1	-	<0.1

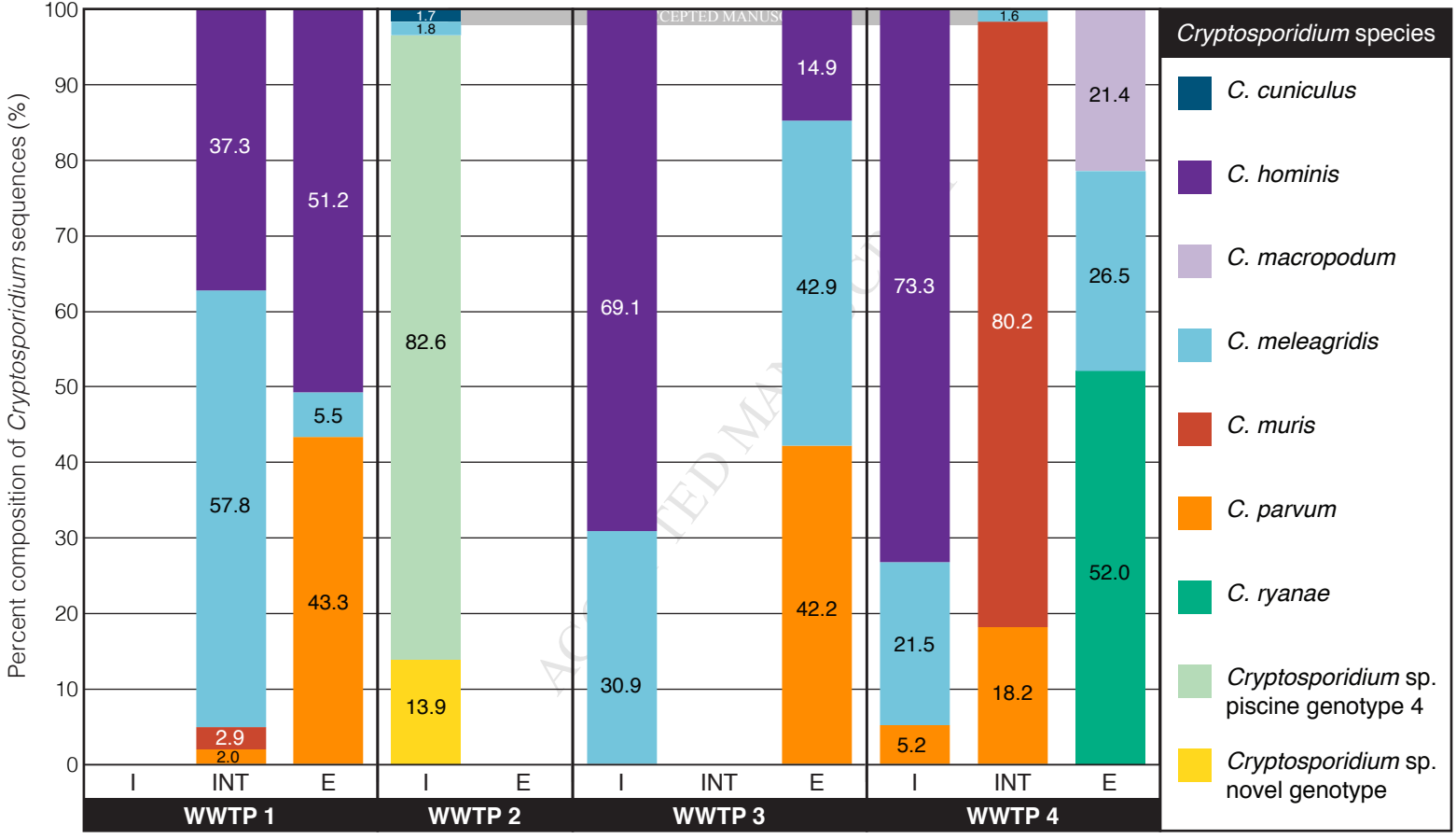
[a]92.4% similar to *Entamoeba bangladeshi* and *E. moshkovskii*
[b]91.4% similar to *Entamoeba ecuadoriensis*
[c]96.2% similar to *E. moshkovskii*
[d]100% similar to *Entamoeba dispar*, *Entamoeba histolytica* and *Entamoeba nuttalli*
[e]93.6% similar to *Blastocystis* sp. ST4
[f]96.8% similar to *Blastocystis* sp. ST4





WWTPs	Influent	Intermediate	Effluent
WWTP 1	Sewerage System	Maturation Ponds	WSPs
	n=2	*n*=2	*n*=4
WWTP 2	Sewerage System	Secondary Pond	WSPs
	n=2	*n*=0	*n*=4
WWTP 3	Sewerage System	Facultative Pond	WSPs
	n=2	*n*=2	*n*=2
WWTP 4	Sewerage System	Oxidation Ditch	Sedimentation Tank
	n=2	*n*=2	*n*=2




Percent composition of Cryptosporidium sequences (%)
100
90
80
70
60
50
40
30
20
10
0
I
INT
E
I
E
I
INT
E
I
INT
E
WWTP 1
WWTP 2
WWTP 3
WWTP 4
37.3
57.8
2.9
2.0
51.2
5.5
43.3
1.7
1.8
82.6
13.9
69.1
30.9
14.9
42.9
42.2
73.3
21.5
5.2
1.6
80.2
18.2
21.4
26.5
52.0
Cryptosporidium species
C. cuniculus
C. hominis
C. macropodum
C. meleagridis
C. muris
C. parvum
C. ryanae
Cryptosporidium sp. piscine genotype 4
Cryptosporidium sp. novel genotype
Wastewater treatment plant and treatment stage

Highlights

- Western Australian WWTPs were studied for faecal pathogens with eukaryotic 18S NGS
- Stabilisation ponds and activated sludge treatment technologies were assessed
- Influent had the highest percent compositions of intestinal parasites
- Six *Blastocystis* subtypes and four *Entamoeba* species were identified
- Nine *Cryptosporidium* species/genotypes were only detected by *Cryptosporidium*-specific NGS