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

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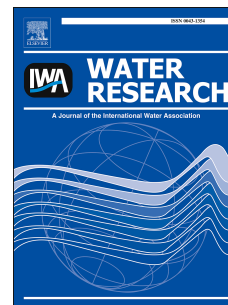
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1 Identification of eukaryotic microorganisms with 18S rRNA next-generation
2 sequencing in wastewater treatment plants, with a more targeted NGS approach
3 required for *Cryptosporidium* detection

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25 **Abstract**

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

47

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

50

51 1. Introduction

52

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

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

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

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

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

83

84 **2. Methods**

85

86 **2.1 Study sites and sample collection**

87

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

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

104

105 **2.2 DNA isolation**

106

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

112

113 **2.3 Next-generation sequencing library preparation**

114

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

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

130

131 **2.4 18S Bioinformatic analysis**

132

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

147

148 **2.5 *Giardia*-specific PCR**

149

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

156

157 **2.6 *Cryptosporidium* 18S PCR amplification and Sanger sequencing analysis**

158

159 Due to its importance as a pathogen in wastewater (Zahedi et al., 2018a),
160 *Cryptosporidium*-specific 18S NGS was conducted to identify any *Cryptosporidium* sequences
161 in the samples. All WWTP samples (*n* = 26) were screened for the presence of *Cryptosporidium*
162 18S using nested primers that amplify ~825 bp products, as previously described (Xiao et al.,
163 1999). The amplified DNA from secondary PCRs were separated by gel electrophoresis and
164 purified for Sanger sequencing using an in-house filter tip method (Yang et al., 2013). Purified
165 PCR products were sequenced independently on an ABI Prism™ Dye Terminator Cycle
166 Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's
167 instructions. Sanger sequencing chromatogram files were imported into Geneious Pro 8.1.6
168 (Kearse et al., 2012), edited, analysed and aligned with reference sequences from GenBank
169 using Clustal W (<http://www.clustalw.genome.jp>). NTCs were included alongside all PCRs to
170 detect contamination.

171

172 **3. Results**

173

174 **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

200 for WWTP 1 (16.1%), WWTP 3 (14.8%) and WWTP 4 (12.8%), and intermediate for WWTP 4
201 (20.8%); Ciliophora in the influent (11.7%) and effluent (39.4%) for WWTP 2, effluent for
202 WWTP 3 (22.9%), and intermediate (35.2%) and effluent (51.1%) for WWTP 4; and
203 Euglenozoa in the influent (44.6%) and effluent (31.3%) for WWTP 2, intermediate (19.5%) and
204 effluent (37.6%) for WWTP 3, and effluent for WWTP 4 (21.4%) (Figure 3). More specific
205 levels of classification with the top BLAST hit GenBank® accession numbers and percent
206 similarities for each ZOTU are provided in Supplementary File B.1. The most abundant 18S
207 sequences in the ExCs and NTCs were identified as alveolates, fungi (Ascomycota and
208 Basidiomycota) and green algae (Charophyta) (Supplementary File B.1).

209

210 3.2.2 Prevalence and composition of intestinal parasites

211

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

218 All *Blastocystis* sp. subtypes (STs) 1-4, 6, 8, and potentially two novel genotypes
219 (*Blastocystis* sp. ZOTU 1064 and ZOTU 1314) were identified in the influent for WWTP 3 and
220 WWTP 4, with percent sequence compositions ranging from $<0.1\%$ for *Blastocystis* ST6 to
221 7.9% for *Blastocystis* sp. ST1 in WWTP 3, and $<0.1\%$ for *Blastocystis* sp. ST6, *Blastocystis* sp.
222 ZOTU 1064 and ZOTU 1314 to 2.6% for *Blastocystis* sp. ST1 in WWTP 4. All STs and
223 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 $\geq 94.0\%$ 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.

249

250 **3.2.3 Prevalence and composition of eukaryotic bioindicators**

251

252 Bacterivorous eukaryotes were detected in all WWTPs, predominantly in the effluent
253 (8.8% in WWTP 1 – 51.1% in WWTP 4), the influent of WWTP 2 (11.7%) and intermediate of
254 WWTP 4 (35.2%). In general, fungal communities were present at very low levels across the
255 WWTPs, with the exception of the influents (15.8% in WWTP 1, up to 12.8% in WWTP 3 and
256 up to 9.1% in WWTP 4) and the intermediate stages of WWTP 3 (8.1%) and WWTP 4 (5.6%).
257 Green algae were detected in all WWTPs with the highest levels in WWTP1 effluent (53.2%),
258 WWTP 2 and 4 influents (16.1 and 48.1% respectively) and WWTP 3 intermediate stage
259 (25.6%). Rotifers were also present across all WWTPs with the highest prevalence in WWTP 3
260 effluent (27.9%) (Table 5).

261

262 **3.3 *Cryptosporidium* sp.**

263

264 Of the 26 samples, a *Cryptosporidium*-specific 18S nested PCR produced amplicons with
265 the correct band size (confirmed by gel electrophoresis) for 20 samples, and Sanger sequencing
266 identified *Cryptosporidium hominis* in one sample (WWTP 4-4). The remaining 19 samples
267 produced either mixed chromatograms or non-specific sequences (e.g. *Colpodella* sp., which
268 were also detected with eukaryotic 18S NGS). The *Cryptosporidium*-specific 18S NGS analysis
269 identified a total of nine *Cryptosporidium* species across the four WWTPs: *Cryptosporidium*
270 *hominis*, *Cryptosporidium parvum*, *Cryptosporidium meleagridis*, *Cryptosporidium muris*,
271 *Cryptosporidium cuniculus*, *Cryptosporidium macropodum*, *Cryptosporidium ryanae*,
272 *Cryptosporidium* sp. piscine genotype 4 and a potentially novel *Cryptosporidium* sp. piscine
273 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 ($\leq 0.2\%$). 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 $\geq 0.9\%$
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.,

324 2012). Like *Endolimax*, *Iodamoeba* has generally been considered non-pathogenic, but some
325 reports have found *Iodamoeba butschlii* infections in children and immunocompromised patients
326 (Waywa et al., 2001; Faulkner et al., 2003). The *Iodamoeba* sequences obtained in this study
327 were either 99.1% or 100% similar to *Iodamoeba* sp. isolated from human faeces (JN635741).

328 The percent sequence composition of *Blastocystis*, *Entamoeba*, *Endolimax*, *Iodamoeba*
329 and *E. vermicularis* was overall higher in the influent and lower or not detected in the
330 intermediate and effluent stages. It is possible that the wastewater treatment processes have
331 reduced the faecal pathogen abundance compared to the influent. However, the number of
332 sequences obtained by NGS does not represent the number of microorganisms present. A
333 number of factors affect sequence composition, including PCR amplification bias (Hong et al.,
334 2009), sequencing depth and copy number variation in the 18S gene. Quantitative assessments
335 (e.g. counting parasite numbers) at different treatment stages are required to accurately
336 determine the efficacy of pathogen removal during the water treatment process.

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

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

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

354 Although the Euk1391F/EukBr primers from the Earth Microbiome Project's protocol
355 were designed to amplify the eukaryotic 18S V9 region ([www.earthmicrobiome.org/protocols-](http://www.earthmicrobiome.org/protocols-and-standards/18s/)
356 [and-standards/18s/](http://www.earthmicrobiome.org/protocols-and-standards/18s/)), the present study found that the primers cross-reacted with archaeal and
357 bacterial 16S and were only slightly more biased towards the amplification of 18S (60% of total
358 pre-processed reads). This may be due to the design of the forward primer (Euk1391F), which is
359 very similar (only 2 bp downstream) to the universal (16S and 18S) primer 1389F designed by
360 Amaral-Zettler et al. (2009). Our findings contrast with the study by Maritz et al. (2017), which
361 used the same primers with NGS on sewage samples and reported "low numbers" of Archaea
362 and Eubacteria amplification with Euk1391F/EukBr. In the present study, the mammalian
363 blocking primer did work as expected, as only 0.03% of the processed sequences were
364 mammalian 18S. Maritz et al. (2017) also compared the Euk1391F/EukBr (with and without the
365 mammalian blocking primer) to the TAREuk454FWD1/TAREukREV3 eukaryotic 18S primers
366 (Stoeck et al., 2009), which target the 18S V4 region, and reported that the
367 TAREuk454FWD1/TAREukREV3 primers used for NGS resulted in sequences with higher
368 variability and taxonomic accuracy (Maritz et al., 2017). Such results have also been shown by
369 another study (Pawlowski et al., 2011). This suggests that the Euk1391F/EukBr primers are less
370 suitable for taxonomic resolution, particularly due to the short sequence length (average length
371 of 107 bp following adapter removal and primer trimming). The significant amount of 16S
372 amplification that occurred in the current study reduced the sequencing depth of 18S (only 17/26
373 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

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

411 Despite the lack of sensitivity for pathogen detection using the Euk1391F/EukBr primers
412 with 18S NGS, an advantage of the method is that it allowed for other groups of eukaryotes to be
413 identified, some of which are bioindicators of water treatment quality. The dominant groups of
414 eukaryotes detected in the WSPs varied; for example, there was a high percent sequence
415 composition of Chlorophyta (green algae) in the intermediate and effluent stages of WWTP 1
416 (53.2-61.1%), whereas the sequence composition of green algae was lower in the same treatment
417 stages for WWTP 2 and WWTP 3 (2.3-25.6%). Chlorophyta are important components of
418 WWTPs and remove nutrients including phosphorus and nitrogen and can improve the final
419 effluent quality through “natural disinfection and incorporation of other contaminants, such as
420 heavy metals, pharmaceuticals and endocrine disrupters” (Abinandan and Shanthakumar, 2015;
421 Schulze et al., 2017). The reason for the lower number of Chlorophyta sequences in WWTP 2
422 and 3 is unknown but may have been affected by PCR amplification bias. The intermediate and
423 effluent stages of the activated sludge plant (WWTP 4) had similar dominant groups of

424 eukaryotes as WSPs WWTP 2 and WWTP 3: Ciliophora (22.9-51.1%) and Euglenozoa (19.5-
425 37.6%) (Figure 3). Previous studies on activated sludge plants have also identified many species
426 of free-living protozoa, e.g. 160/228 protozoan species detected in activated sludge plants have
427 been identified as Ciliophora (Curds, 1975). A drawback of the activated sludge treatment
428 process is high sludge (bacteria) production, which is difficult to process with high nitrogen,
429 phosphate and heavy metal concentrations (Ratsak et al., 1996). Protozoa that predate on
430 bacteria are beneficial in activated sludge plants as this directly helps to reduce sludge. Well-
431 functioning activated sludge plants have communities dominated by bacterivorous free-
432 swimming ciliates (phylum Ciliophora) that swim in the liquor phase (Curds, 1966), crawling
433 ciliates that move on the surface of sludge floc (e.g. the hypotrichs *Aspidisca* and *Euplotes*), and
434 sessile ciliates that attach to the sludge floc (e.g. the peritrichs *Carchesium*, *Epistylis*,
435 *Opercularia coarctata*, *Vorticella* and *Zoothamnium*) (Madoni, 2011). In the current study,
436 within the Ciliophora, the subclass Peritrichia dominated, which is the most important group of
437 bacterivorous ciliates in WWTPs and play a central role in effluent clarification, reducing
438 biochemical oxygen demand (BOD) and turbidity (Madoni, 2003). The hypotrichs *Euplotes* sp.
439 and the peritrichs *Epistylis* sp., and *Vorticella* sp. were detected in the intermediate and effluent
440 stages for the activated sludge plant, WWTP 4, and were also detected in the WSPs
441 (Supplementary File B.1). Other bacterivorous eukaryotes found were *Leptomyxa* sp., which is
442 typically found in soil (Del Valle et al., 2017), the free-living nanoflagellates *Trimastix* sp.
443 (Bernard et al., 2000), the aquatic fungi Cryptomycota, which parasitise phytoplankton such as
444 diatoms, green algae, dinoflagellates and cyanobacteria (Rojas-Jimenez et al., 2017), and
445 members of the phylum Loukozoa, which are free-living heterotrophs found in marine or
446 freshwater environments (Simpson, 2016). The presence of so many bacterivorous eukaryotes
447 indicates the lower technology WSPs, as well as the activated sludge plant, are functioning well,
448 in a state where protozoa are predated on bacteria and reducing sludge quantities.

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

461

462 **5. Conclusions**

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

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

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

497

498 **Appendices**

499

500 Appendix A. Supplementary Data

501 Supplementary File A.1.

502

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510

511 Figure Legends

512

513 Figure 1. WWTP localities and different treatment stages sampled.

514

515 Figure 2. Alpha rarefaction plot of sequencing depth and ZOTUs detected. The plot was
516 generated with the R package vegan (Oksanen et al., 2018) using R software (R Core Team,
517 2013).

518

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

523

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).

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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
	Loukzoza	-	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)	
				7-Sep-2015; Dry	WWTP 1-4	Influent
					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)	
				13-Jul-2015; Winter	WWTP 2-4	Influent
					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. **ACCEPTED MANUSCRIPT**

Target organisms	Primer names	Primer sequences (5'-3')	Product size (bp)	Annealing Temperature (°C)	References
<i>Cryptosporidium</i> sp.	18S iF	AGTGACAAGAAA TAACAATACAGG	~298	60	Morgan et al., 1997
	18S iR	CCTGCTTTAAGCA CTCTAATTTTC			
Eukaryotes	Euk1391F	GTACACACCGCC CGTC	~107 ^a	65	www.earthmicrobiome.org/protocols-and-standards/18s/
	EukBr	TGATCCTTCTGCA GGTTCACCTAC			
Mammals	Mammalian blocking primer Mammal_block_I-short_1391f	GCCCGTCGCTACT ACCGATTGG44444 TTAGTGAGGCC3 ^b	-		

^aAverage sequence length after adapter removal and primer trimming^b3 = C3 spacer; 4 = deoxyinosine

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

Eukaryotic 18S NGS sequences						
Statistics	Raw (unprocessed)	Pre-processed ^a	Processed 18S sequences ^b			
	Grand total (<i>n</i> = 36)		Samples (<i>n</i> = 26)	Extraction controls (<i>n</i> = 6)	NTCs (<i>n</i> = 4)	Grand total (<i>n</i> = 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
<i>Cryptosporidium</i> -specific 18S NGS sequences						
Statistics	Raw	Pre-processed ^a	Processed <i>Cryptosporidium</i> 18S sequences ^c			
	Grand total (<i>n</i> = 33)		Samples (12/26) ^d	Extraction controls (<i>n</i> = 6)	NTCs (<i>n</i> = 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	

^aMerged, quality filtered sequences with singletons, chimeras and contaminant ZOTUs removed

^bMerged, quality filtered sequences with singletons, chimeras, contaminant ZOTUs, bacterial and archaeal 16S sequences removed

^cMerged, quality filtered sequences with singletons, contaminant ZOTUs and non-*Cryptosporidium* sequences removed

^dNumber of *Cryptosporidium*-positive samples over total sample number

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

Phylum	ZOTU No.	Accession number	Species	Top BLAST hit accession	Similarity (%)	WWTP 1			WWTP 2		WWTP 3			WWTP 4		
						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	<i>Endolimax</i> spp.	AF149916	94.0-99.1	0.7	<0.1	-	-	-	0.6	<0.1	-	0.1	-	<0.1
	485	MH623050	<i>Entamoeba coli</i>	AF149915	100	2.2	-	-	-	-	0.7	-	-	0.2	<0.1	<0.1
	1563	MH623056	<i>Entamoeba hartmanni</i>	KX618191		<0.1	-	-	-	-	0.2	-	-	0.1	-	-
	2095	MH623061	<i>Entamoeba moshkovskii</i>	KP722601		<0.1	-	-	-	-	0.1	-	-	<0.1	<0.1	-
	538	MH623051	<i>Entamoeba polecki</i>	LC082304		0.8	-	<0.1	-	-	-	-	-	-	-	-
	1622	MH623057	<i>Entamoeba</i> sp. ZOTU 1622 ^a	KP722601		93.5	-	-	-	-	-	<0.1	-	-	0.2	<0.1
	2260	MH623063	<i>Entamoeba</i> sp. ZOTU 2260 ^b		91.7	<0.1	-	-	-	-	<0.1	-	-	<0.1	<0.1	-
	405	MH623049	<i>Entamoeba</i> sp. ZOTU 405 ^c		96.2	<0.1	-	-	-	-	0.4	-	-	2.9	<0.1	-
	832	MH623052	<i>Entamoeba</i> sp. ZOTU 832 ^d	LC041205	100	0.4	-	<0.1	-	-	0.4	-	-	0.1	<0.1	<0.1
	2906	MH623069	<i>Iodamoeba</i> sp. ZOTU 2906	JN635741	100	<0.1	-	-	-	-	-	-	-	<0.1	-	-
	3246	MH623073	<i>Iodamoeba</i> sp. ZOTU 3246	JN635741	99.1	0.1	-	-	-	-	-	-	-	-	-	-
Nematoda	327	MH623047	<i>Enterobius vermicularis</i>	JF934731	100	1.8	-	-	-	-	-	-	-	-	-	-
Sarcomastigophora	124	MH623043	<i>Blastocystis</i> sp. ST1	KM213500	100	6.4	<0.1	<0.1	-	<0.1	7.9	0.1	-	2.6	-	<0.1
	244	MH623045	<i>Blastocystis</i> 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	<i>Blastocystis</i> sp. ST3	KX618192		3.6	<0.1	<0.1	<0.1	<0.1	1.7	-	-	1.3	<0.1	<0.1

288	MH623046	<i>Blastocystis</i> 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	<i>Blastocystis</i> sp. ST6	EU445485		0.2	-	-	-	-	<0.1	-	-	<0.1	-	-
345	MH623048	<i>Blastocystis</i> sp. ST8	AB107971		1.4	<0.1	<0.1	-	-	0.7	-	-	0.2	<0.1	<0.1
1064	MH623053	<i>Blastocystis</i> sp. ZOTU 1064 ^e	JN682513	94.4	<0.1	-	-	-	-	0.6	-	-	<0.1	-	-
1314	MH623055	<i>Blastocystis</i> sp. ZOTU 1314 ^f	KU939393	96.8	-	-	-	-	-	0.4	-	-	<0.1	-	<0.1

^a92.4% similar to *Entamoeba bangladeshi* and *E. moshkovskii*

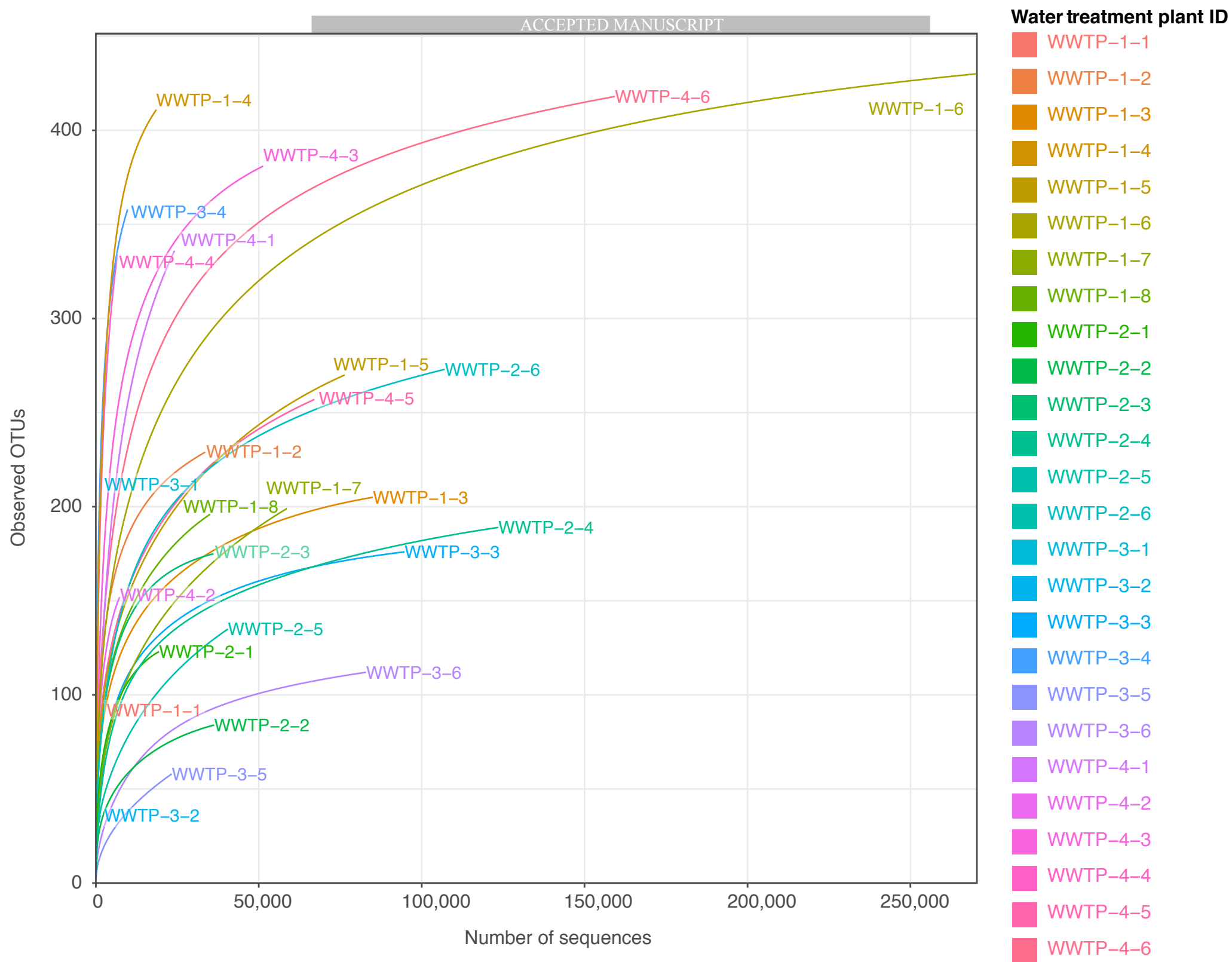
^b91.4% similar to *Entamoeba ecuadoriensis*

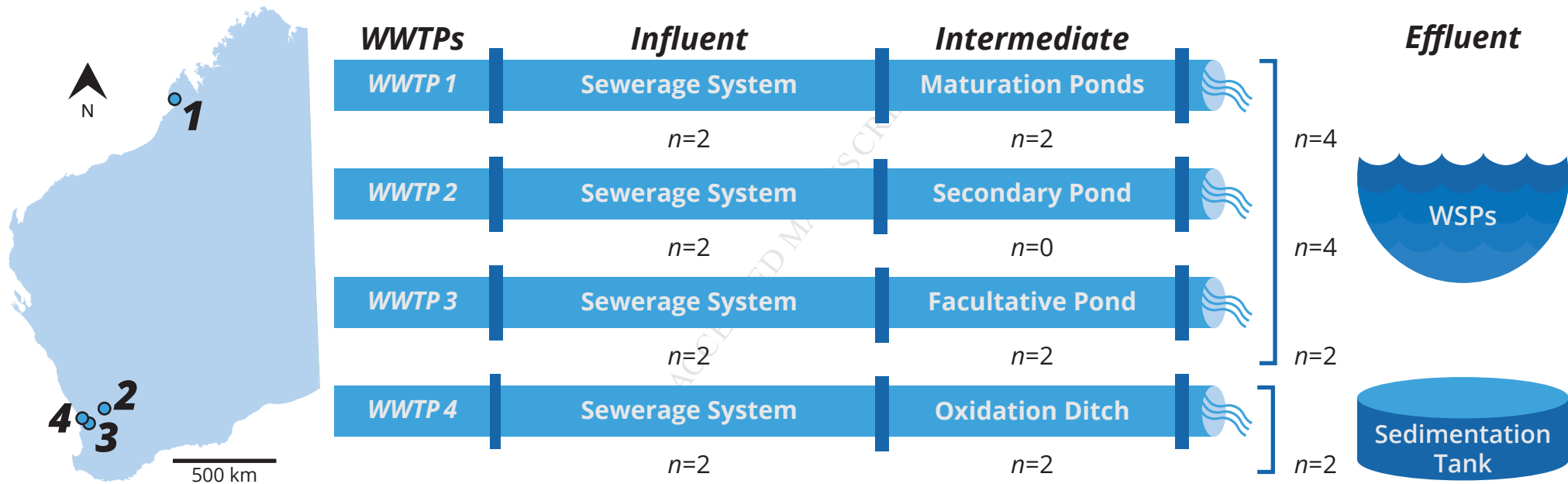
^c96.2% similar to *E. moshkovskii*

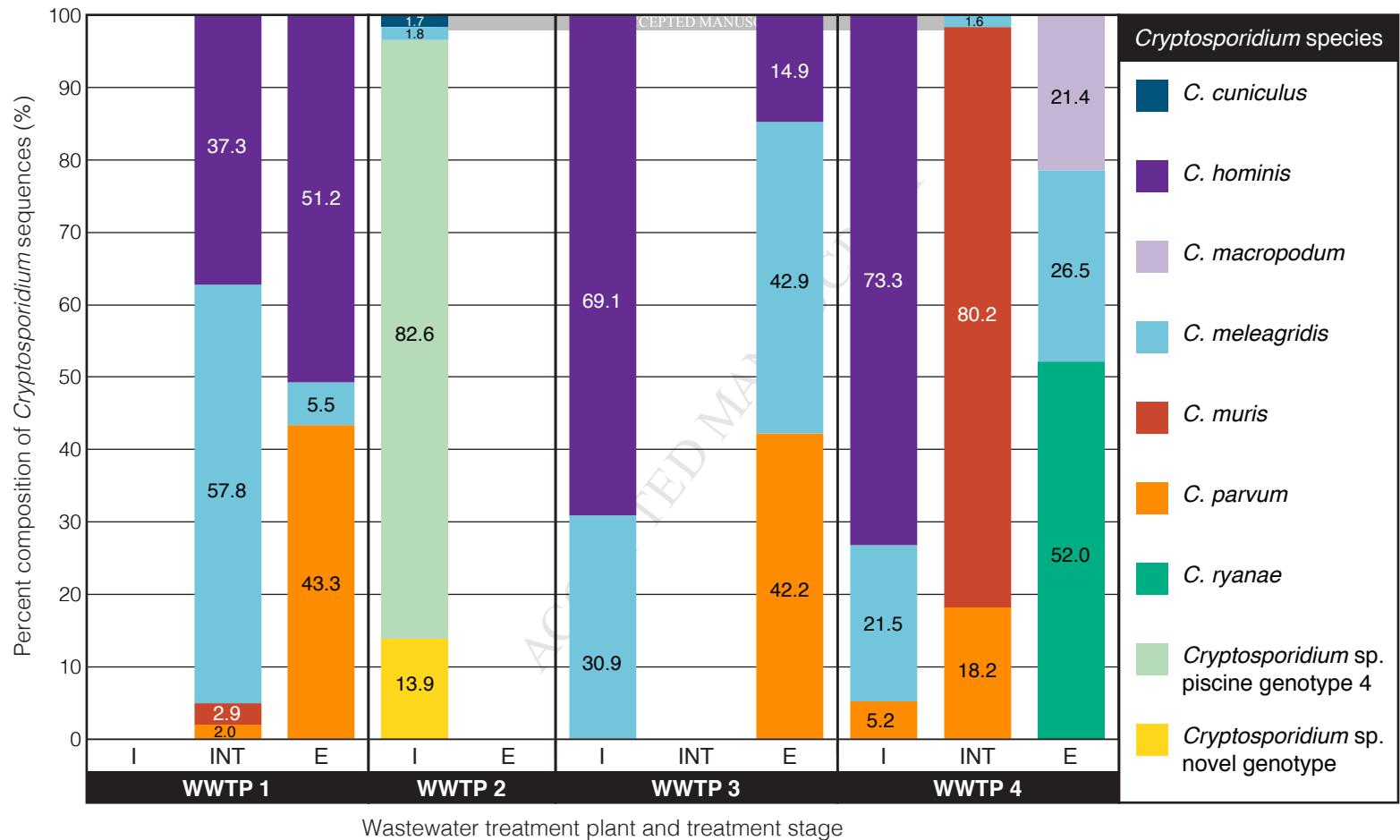
^d100% similar to *Entamoeba dispar*, *Entamoeba histolytica* and *Entamoeba nuttalli*

^e93.6% similar to *Blastocystis* sp. ST4

^f96.8% similar to *Blastocystis* sp. ST4







1 **Highlights**

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