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Soler-Serena, P.; Moreno-Mesonero, L.; Zornoza-Zornoza, AM.; Macián Cervera, VJ.; Moreno Trigos, MY. (2021). Characterization of eukaryotic microbiome and associated bacteria communities in a drinking water treatment plant. Science of The Total Environment. 797:1-14. https://doi.org/10.1016/j.scitotenv.2021.149070

The final publication is available at

https://doi.org/10.1016/j.scitotenv.2021.149070

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Additional Information

1 **Characterization of eukaryotic microbiome and associated bacteria communities in a drinking**

2 **water treatment plant**

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4

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23 **Abstract**

24 The effectiveness of drinking water treatment is critical to achieve an optimal and safe drinking
25 water. Disinfection is one of the most important steps to eliminate the health concern caused
26 by the microbial population in this type of water. However, no study has evaluated the changes
27 in its microbiome, specially the eukaryotic microbiome, and the fates of opportunistic pathogens
28 generated by UV disinfection with medium–pressure mercury lamps in drinking water treatment
29 plants (DWTPs). In this work, the eukaryotic community composition of a DWTP with UV
30 disinfection was evaluated before and after a UV disinfection treatment by means of Illumina
31 18S rRNA amplicon-based sequencing. Among the physicochemical parameters analysed, flow
32 and nitrate appeared to be related with the changes in the eukaryotic microbiome shape.

33 Public health concern eukaryotic organisms such as *Blastocystis*, *Entamoeba*, *Acanthamoeba*,
34 *Hartmannella*, *Naegleria*, *Microsporidium* or *Caenorhabditis* were identified.

35 Additionally, the relation between the occurrence of some human bacterial pathogens and the
36 presence of some eukaryotic organisms has been studied. The presence of some human
37 bacterial pathogens such as *Arcobacter*, *Mycobacterium*, *Pseudomonas* and *Parachlamydia*
38 were statistically correlated with the presence of some eukaryotic carriers showing the public
39 health risk due to the bacterial pathogens they could shelter.

40

41 Keywords :

42 DWTP microbiome, Eukaryotes, waterborne bacteria, UV disinfection, amplicon-based
43 sequencing

44 **1. Introduction**

45 Drinking water treatment processes, control of physicochemical parameters and disinfection are
46 critical to public health. Although drinking water treatment plants (DWTPs) have demonstrated
47 sufficient capacity to remove particles, chemicals, and microorganisms from water sources,
48 some microorganisms remain and form the characteristic microbiome of drinking water.
49 Changes in the microbiome shape could produce an imbalance in the microbial community, thus
50 generating adverse effects such as pathogen increase, corrosive phenomena or appearance of
51 odours and flavours which could affect the effectivity of the water treatment. The existing
52 studies which focus on the microbial ecology along drinking water treatment processes reveal a
53 great diversity of bacterial and eukaryotic communities, as well as substantial community shifts
54 during filtration and disinfection steps (Pinto et al., 2012; Lautenschlager et al., 2014; Wang et
55 al., 2014). However, there is still a lack of information of the effects of each drinking water
56 treatment process and physicochemical parameters regarding the microbial composition,
57 especially for facilities employing multi-step treatment processes (Lautenschlager et al., 2014).

58 The use of UV disinfection systems for disinfection purposes has been increased in DWTPs due
59 to its advantages over other systems. UV radiation has the ability to penetrate cell walls and
60 cause damage to nucleic acids (i.e., DNA and RNA), which leads to the inability of the cells to
61 replicate and thus causes their death or inactivation (Snicer et al., 2000; Betancourt and Rose,
62 2004) although this state is difficult to define in environment. UV radiation does not depend
63 on the use of chemical additives; it does not have disinfection by-products, it is not corrosive,
64 has great efficiency to eliminate resistant microorganisms to disinfection such as protozoa,
65 requires relatively short contact time and has fewer operating costs (Betancourt and Rose,
66 2004). However, its efficacy depends on many factors, such as water source, water quality, type
67 of UV lamps, UV wavelength and intensity, time of exposure, the reactor structure, interference
68 of turbidity and hydraulic conditions (Nizri et al., 2017).

69 There are many bacterial and eukaryotic microorganisms, mainly pathogenic, with resistance or
70 repair mechanisms that can reduce the effectiveness of UV inactivation or cause the need to
71 sporadically increase the required dose (Rochelle et al., 2004; Bichai et al., 2008). Those
72 opportunistic pathogens which include bacterial species such as *Legionella pneumophila* and
73 free-living amoebae (FLA) such as *Acanthamoeba* spp. have attracted global increasing attention
74 due to the serious health problems they can cause and the involvement of drinking water
75 systems as their transmission routes (Marciano-Cabral et al., 2010; Moreno et al., 2019).
76 Moreover, the presence of FLA in drinking water sources increases the possibility of human
77 infections due to their bacterial hosts, such as *Helicobacter*, *Legionella* or *Mycobacterium*,
78 among others (Thomas and Ashbolt al., 2011; Moreno-Mesonero et al., 2017).

79 One of the most important mechanisms to evade an adequate UV disinfection process, and at
80 the same time less studied, is the association of bacteria with a superior organism, generally
81 constituting a relationship of symbiosis or parasitism. This relationship causes the
82 microorganism to be protected by the superior organism, such as amoebae or rotifers, among
83 others (Bichai et al., 2008). Since it has been proved that certain microorganisms survive to
84 chemical disinfectants through this type of interaction, it is considered that UV radiation can be
85 a more convenient disinfection mechanism if it can inactivate the microorganisms that provide
86 shelter to others (Bichai et al., 2008).

87 Considering the typical low abundances of pathogenic protozoa, especially in drinking water,
88 large volumes of water along with the use of sensitive molecular techniques are necessary to
89 characterize this type of water samples. Advances in the sequencing technology have enabled
90 the use of high-throughput sequencing of microbial communities which provide more detailed
91 microbial community structure analysis with higher taxonomic resolution (Andersson et al.,
92 2010). The use of Illumina MiSeq sequencing data generated by rRNA amplicon-based targeted
93 sequencing is now commonplace in water microbial communities' studies. Few studies have
94 applied the 16S rRNA amplicon sequencing to study the microbial community in drinking water

95 systems (Bautista de los Santos et al., 2016; Xu et al., 2017; Zhao et al., 2020) and scarce have
96 studied the eukaryotic communities by 18S rRNA amplicon sequencing (Lin et al., 2014; Ting et
97 al., 2021). Despite the importance of the occurrence of some eukaryotic organisms in drinking
98 water systems, either to be pathogenic or to be pathogenic bacteria sheltering eukaryotes, to
99 our knowledge, there are no reports regarding the microbiome characterization of this
100 community in UV disinfected water and there are only a few reports in chlorine disinfected
101 water (Inkinen et al., 2019).

102 Thus, the objectives of this study have been to characterize the eukaryotic microbiome and their
103 associated bacteria in a DWTP before and after UV disinfection by Illumina MiSeq 18S and 16S
104 rRNA amplicon sequencing. Moreover, the relationship between the bacterial community
105 (mainly waterborne pathogens) and the eukaryotic community, and their role as potential hosts,
106 is studied, as well as the possible influence of the physicochemical parameters.

107

108 **2. Material and methods**

109 **2.1. Drinking water treatment process**

110 The DWTP samples were collected from a plant located in Valencia province (Spain). The water
111 influent is a mix of Turia River and Júcar-Turia canal. This DWTP has the capacity to treat up to
112 3.2 m^3/s. The nominal population served by the DWTP is around 800,000 inhabitants.

113 The plant has a conventional treatment consisting of: (a) pre-treatment (roughing and pre-
114 oxidation disinfection by chlorine gas and/or chlorine dioxide; (b) clarification (coagulation-
115 flocculation-decantation); (c) filtration on activated carbon filters; and (d) UV disinfection with
116 medium-pressure mercury lamps, and, prior to distribution, chlorine disinfection with a final
117 residual concentration of 1 mg/L.

118 This preference is easily justifiable based on greater health assurance and prevention of in-
119 process water contamination; even more so when the application of UV radiation is carried out
120 in the later stages of treatment. The UV treatment is located after the filtration stage and before
121 the galleries or tanks (Fig. S1). The reason for locating the lamps at this point is that the water
122 must have a very low turbidity, otherwise the lamps would become dirty, and disinfection would
123 be ineffective.

124 The reactor used is a WEDECO UV reactor, model K143 12/7. The lamps are medium-pressure
125 mercury lamps, model SLR32143/4pHP from WEDECO (USA). The minimum dose of UV light
126 applied to the treatment plant is 400 J/m^2 and measurements are taken every minute, resulting
127 in a final average of 420 J/m^2.

128 **2.2. Water samples and processing**

129 The sampling campaign (14 samplings) was conducted during a 15-month period which covered
130 all seasons. A total of 28 samples were collected, which comprised14 samples after the carbon
131 filter treatment (before UV treatment) and 14 samples after the UV disinfection treatment but
132 prior to chlorine addition. Samples' names include the number of the sampling in which they

133 were taken (Samplings 1-14) followed by the last treatment they underwent (C: active carbon
134 filters; UV: UV disinfection treatment) (Table S1).

135 A total of 200 litres of water after the active carbon filter treatment and after the UV disinfection
136 treatment were filtered through Envirochek® HV capsules (1 µm pore size membrane) (Pall
137 Gelman Laboratory, Ann Arbor, MI, USA) at a continuous flow rate of up to 2 L/min, following
138 the procedures described in Method 1623.1 of the U.S. Environmental Protection Agency (EPA)
139 (USEPA, 2012). A great volume of water was analysed to concentrate the maximum number of
140 protozoa. Envirochek® HV capsules were developed for the protozoa *Cryptosporidium* and
141 *Giardia,* however, this method is suitable to recover other protozoa (Zuckerman and Tzipori,
142 2006). Briefly, membranes were pre-treated using 150 mL of 5% sodium hexametaphosphate.
143 Then, samples were filtered and thereafter, capsules were filled with 250 mL of elution buffer,
144 placed on a laboratory shaker, and vigorously shaken to elute any captured protozoa. Total
145 eluted buffer was concentrated by centrifugation at 1,800 g for 15 min.

146 **2.3. Physicochemical and microbiological analysis**

147 The physicochemical parameters temperature, pH, conductivity, turbidity, colour, nitrites,
148 nitrates and ammonium were measured. pH measurement was carried out using CRISON GLP22
149 pH meter (APHA, 2005). Conductivity was determined using a potentiometric system (UNE-EN
150 27888). Turbidity was determined using the turbidity meter HI 88703 (Hanna Instruments Ltd.,
151 UK), according to the manufacturer's instructions. Water colour was measured using the UV/Vis
152 DR-5000 spectrophotometer (Hach-Lange, Germany) according to UNE-EN 7887 normative.
153 Nitrites were determined by spectrophotometry using the UV-1601 spectrophotometer
154 (Shimadzu Corporation, Japan) following the standard method SM 4500-NO2-B (APHA, 2005).
155 Nitrates were measured using the UV/Vis DR-5000 spectrophotometer (Hach-Lange, Germany)
156 following the standard method SM 4500-NO3-B. Finally, ammonium was determined
157 spectrophotometrically by means of the Nesslerization technique following ASTM D1426

158 Method A (APHA, 2005) and using the UV-1601 spectrophotometer (Shimadzu Corporation,
159 Japan). The flow treated by the plant during the study was measured with the flowmeter MIBUS-
160 RISONIC 2000 (Rittmeyer).

161 The microbiological parameters to control the quality of water were total coliforms, *Escherichia*
162 *coli*, *Clostridium perfringens* and total aerobic bacterial counts (Spanish normative RD 140/2003)
163 For this purpose, 100 mL of each water sample were filtered through a sterile 0,45 µm pore size
164 nitrocellulose membrane (Millipore, Billerica, MA, USA) for each analysis, according to the
165 different ISO standards. The analysis of the total coliforms and *E. coli* indicators were carried out
166 according to the standard method UNE-EN ISO 9308-2:2014 using the Colilert culture medium
167 (IDEXX Laboratories Inc., Westbrook, ME, USA). *C. perfringens* detection, including its spores,
168 was carried out according to the standard method UNE-EN ISO 14189:2013. Total aerobic
169 bacterial counts were obtained by the pour-plate technique at 35 ± 0,5 °C for 70 ± 2 h, which is
170 appropriate for heterotrophic bacteria using TSYE agar (HiMedia, SM 9215B) (UNE-EN ISO
171 6222:1999).

172 **2.4. DNA extraction and sequencing**

173 Total DNA was extracted from the concentrated water samples using FastDNA™ SPIN Kit for Soil
174 (MP Biomedicals, Irvine, CA, USA), performing the homogenization step in the FastPrep-24®
175 instrument (MP Biomedicals, Irvine, CA, USA), following the manufacturer's instructions and
176 finally eluting DNA in 50 µL of elution buffer.

177 To determine the eukaryotic community of the samples, the V4 hypervariable region of the 18S
178 rRNA gene was amplified in all samples using the primers set EUKAF: 5'- GCC GCG GTA ATT CCA
179 GCT C-3' and EUKAR: 5'- CYT TCG YYC TTG ATT RA-3' using the enzyme KAPA HiFi HotStart with
180 GC buffer (KAPA Biosystems, USA) as described by Moreno et al. (2018). To determine the
181 bacterial community associated to eukaryotes, the V3-V4 regions of the 16S rRNA gene were
182 amplified in 18 samples using the recommended set of primers and conditions specified by the

183 16S Metagenomic Sequencing Library Preparation guide (Part # 15044223 Rev. B) (Klindworth
184 et al*.,* 2013). Both types of DNA amplicon libraries were sequenced on an Illumina MiSeq
185 sequencer using the automated cluster generation and paired-end sequencing with dual indexes
186 reads (2 × 300 bp) at FISABIO sequencing service (Valencia, Spain).

187 **2.5. Bioinformatics and data analysis**

188 Raw Illumina MiSeq sequencing data was analysed using QIIME 1.9.1 (http://qiime.org;
189 Caporaso et al., 2010), applying additional scripts available in Microbiome Helper virtualbox
190 (Comeau et al., 2017). Briefly, forward and reverse reads were merged. Subsequently, stitched
191 reads were filtered by length and quality score (reads with less than 200 bp or a minimum quality
192 score of Q30 over at least 90% of the bp were removed) using FASTX-Toolkit v0.0.14 (Gordon,
193 2009). Reads with any ambiguous bases ("N") were also filtered out. Potential chimeric
194 sequences were screened out using VSEARCH v1.11.1. (Rognes et al., 2016). The remaining
195 sequences were processed using the QIIME's open reference script. Operational Taxonomic
196 Units (OTUs) were defined at 97% genetic similarity cut-off. The PR2 v4.5 Protist Ribosomal
197 Reference database was used to perform the taxonomic assignment for the eukaryotic
198 microbiome (Gillou et al., 2013). In the case of the bacterial microbiome taxonomic assignment,
199 the SILVA v132 ribosomal database was used as a reference (Quast et al., 2013). Alpha diversity
200 indices (Shannon, Simpson and Chao1) and rarefaction curves were calculated with subsampled
201 sequencing data (10,541 and 37,420 sequences for 18S rRNA and 16S rRNA amplicon-based
202 sequencing, respectively) to reduce the effects of different sampling depth.

203 The OTUs distribution of bacterial and eukaryotic data were separately analysed using
204 multivariate routines in the statistical software package PRIMER v7 (Clarke and Gorley, 2015)
205 with PERMANOVA+ add-on (Anderson et al. 2008). Non-metric multidimensional scaling (nMDS)
206 was used to visualize patterns in the treatment stages distribution (active carbon and UV) of the
207 most abundant genera of eukaryotic communities. Prior to analysis, a Bray-Curtis resemblance

208 matrix was created from the biological transformed data (square root transformed) (Bray and
209 Curtis, 1957). On the other hand, microbial community differences between treatment stages
210 were examined using one-way ANOSIM. This analysis was based on the Bray-Curtis similarities
211 between samples and produced a test statistic R, which could range from -1 to 1 and also gives
212 a significance level (P). A near-zero value for R implied no differences between samples (Clarke
213 et al., 2014). Hierarchical clustering (CLUSTER), based on group average linking, was carried out
214 for testing the similarity among OTUs, showing their abundance on a heatmap.

215 The effect of environmental variables (explanatory variables) on the eukaryotes structure, and
216 the analysis of the relationships between carrier eukaryotes (explanatory variables) and
217 pathogenic bacteria were assessed using distance-based linear models (DistLM; McArdle and
218 Anderson, 2001; Anderson et al., 2008). The environmental variables were standardized using
219 the "normalize" routine in PRIMER-E (Clarke and Gorley, 2015) to eliminate their physical units
220 (Legendre and Birks, 2012), and log-transformed. Prior to DistLM, draftman plots and correlation
221 matrices were produced to assess the distribution of each variable and to identify co-correlating
222 environmental variables. Where pairs of variables had a Pearson´s correlation coefficient of 0.85
223 or larger, one of the correlating variables was excluded from the analysis. DistLM was performed
224 with selection base on the AIC_C (corrected Akaike´s information criterion), step-wise selection
225 procedure and 999 permutations, in the case of the environmental variable's ordination model.
226 The AIC_C was devised to handle situations where the number of variables (N) is small compared
227 to the number (v) of predictor variables ($N/v < 40$) (Anderson et al. 2008). In the case of the
228 carrier eukaryotes ordination model, the "Forward" procedure was used with adjusted R^2
229 criterion and 999 permutations. Distance-based redundancy analysis (dbRDA; McArdle and
230 Anderson, 2001; Anderson et al. 2004) was used to allow the visualization of the ordination
231 according to the multivariate regression model previously generated by applying DistLM.

232 To assess the relationship between the response variables (dependents), their Pearson´s
233 correlation coefficients were overlapped with the base variables of the model (explanatory

234 variables). The results were shown in a neural network, using Cytoscape (v3.8.0) (Shannon et al.
235 2003).

236 **3. Results and discussion**

237 **3.1. Physicochemical and microbiological parameters**

238 The water quality parameters for each sample are shown in Table 1. Both, physico-chemical and
239 microbiological parameters were within the recommended national standards for potable water
240 according to the Ministry of Health (RD 140/2003). The physicochemical parameters are stable
241 throughout the study, since the conventional treatment does not modify their values, and only
242 the microbiological parameters are modified after disinfection (ND). Temperature values only
243 showed differences among the samples taken in different seasons of the year. The pH values
244 varied between 7.5 and 7.9 along the samples. These values can fluctuate according to the pH
245 of the rain in equilibrium with atmospheric CO_2 and the same dissolved in the water.
246 Conductivity values ranged between 862 and 1,164 µS/cm, which are typical values of the earth
247 through which the raw water arrives to the DWTP. The turbidity of the samples did not exceed
248 0.5 NTU, except for two samples taken after the active carbon filters, 2C and 3C, which had
249 values of 2.10 and 1.00 NTU, respectively, probably because of an increase of turbidity in raw
250 water. The turbidity was removed by flocculation-sedimentation stage during treatment. As
251 showed, ammonium and nitrites were eliminated after dosing of disinfectant at the start of
252 treatment and at the sampling points, only in samples 1C and 1UV, taken at the same sampling,
253 the results were 0.03 and 0.02 mg/L, respectively. However, these values were below the
254 maximum permitted value (0.5 mg/L for ammonium and 0.1 mg/L for nitrites in the final
255 product), illustrating the effectiveness of the treatment in reducing nitrites and ammonium by
256 the reaction of chlorine and ammonia nitrogen (Hayes-Larson and Mitch, 2010). Nitrates values
257 were affected by the raw water mixture proportion of river and canal water. However, these
258 values were under the maximum permitted value of 50 mg/L (RD 140/2003).

259 The flow rate treated by the plant ranged from 0.35 to 2.13 m3/s along the study. The flow rate
260 depends on the daily demand with a maximum flow rate of 3 m3/s.

261 The microbiological parameters were controlled by culture ISO standard methods. They showed
262 that the microbial quality of water was optimal after the initial disinfection step and before
263 activated charcoal filtration treatment. Only in some samples taken before disinfection with UV
264 lamps and after treatment with activated charcoal, some bacterial colonies were detected by
265 culture, which indicates a possible contamination of the charcoal filters. After treatment with
266 the UV lamps, the standard microbial analysis yielded negative results. The analysis of *C.*
267 *perfringens* and total aerobics yielded negative results for all the samples. The results in Table
268 S1 show that the disinfection carried out at the treatment plant with an initial pre-chlorination
269 and UV disinfection is sufficient to eliminate the microorganisms included in the standards.
270 Although the germicidal effectiveness of UV disinfection processes can be monitored by
271 measuring indicator bacteria using traditional plate-count techniques, the inactivation of
272 specific bacteria or bacterial groups does not guarantee an acceptable degree of inactivation of
273 other waterborne organisms. In most cases, conventional cultivation covers only a minor
274 proportion of the bacteria occurring in a particular habitat. Even bacteria that usually grow on
275 traditional media can lose cultivability after UV exposure despite retaining viability and
276 infectious capacity (Ben Said et al., 2010). Consequently, standard culture-based methods
277 cannot reflect the efficacy of the full-scale UV systems on-site, and additional methods are
278 required to evaluate UV disinfection efficacy.

279 **3.2. Eukaryotic microbiome characterization**

280 In this work, eukaryotic communities were studied before and after UV treatment to
281 characterize the changes in the shape of the eukaryotic microbiome in order to better
282 understand the impact of this group on this ecosystem. After sequencing the 28 samples, a total
283 of 2,321,464 eukaryotic raw reads were obtained. After quality filtering and chimeras screening,

284 2,029,900 reads remained. Following subsampling at 10,541 reads per sample, reads were
285 clustered into 2,451 OTUs (Table S2).

286 The richness and diversity of the samples were evaluated by the alpha diversity indices Shannon,
287 Simpson and Chao1 (Table S4). There were no significant differences in the Alpha-diversity
288 indices between the eukaryotic community before and after UV treatment ($p>0.05$). However,
289 Chao1 index and therefore the observed species, as expected, were higher in the samples before
290 UV treatment, except for the samplings 3, 6 and 10 (Fig. S2).

291 As shown in the nMDS plot, no cluster was observed in eukaryotic communities at phylum and
292 genus levels according to the treatment factor (active carbon and UV) (Fig. 1). Based on these
293 results, it seems that UV treatment do not significantly shape the eukaryotic microbiome of the
294 DWTP. Otterholt and Charnock (2011) observed similar results in a study of the eukaryotic
295 community of UV treated drinking water samples using PCR-denaturing gradient gel
296 electrophoresis (DGGE). They found only a slight variation among the eukaryotic profiles before
297 and after UV treatment. Moreover, Ma et al. (2017) concluded that treatment of drinking water
298 drove the shape of the water microbiome, but they observed by studying the fungal community,
299 that eukaryotes were less influenced by disinfection.

300 Opistokonta was the most frequent group in DWTPs even after disinfection treatments (Inkinen
301 et al., 2019). Opisthokonta (rotifers, nematodes, fungi), Stramenopiles (algae) and Alveolata
302 (ciliates, dinoflagellates), accounted for 84.24 -86.43% of the total eukaryotic microbiome across
303 the samples (Fig. 2, Table S3). Rhizaria relative abundance decreased slightly after UV treatment.
304 The analysis of the most abundant OTUs along the samples, classified at genus level as *Tobrillus*,
305 *Lepidodermella* or *Chromadorea* (Metazoa*),* confirmed a similar taxonomic profile between the
306 microbiome before and after UV treatment. The most abundant OTUs in the DWTP eukaryotic
307 microbiome before and after UV were classified as Metazoa, including sequences belonging to

308 Gastrotricha, Nematoda, Arthropoda or Rotifera phyla, which considerably contribute to the
309 dynamics of the DWTP ecosystem since they feed on bacteria, fungi and protozoa.

310 **3.3. Relationship between the eukaryotic community and environmental variables**

311 The multiple regression analysis (DistLM) test was used to explore the relationship between the
312 eukaryotic species community and the environmental variables (Table S5). A total of 43.72% of
313 the variation in the eukaryotic species assemblage could be explained by the two dbRDA axes.
314 More specifically, dbRDA1 axis explained 40.45% of the total variation in the species assemblage,
315 while dbRDA2 axis only explained 3.27%. The results of the sequential DistLM test showed that
316 NO_3 *c*ontributed with the highest percentage variance explained (p=0.007, 26.96%), followed by
317 the flow (p=0.005, 16.76%). Both variables, NO_3 and flow, were statistically significant. Other
318 studies have already found the correlation between NO_3 and microbial communities. In this
319 sense, Liu et al. (2020) also observed a high correlation between eukaryotic communities and
320 NO_3 concentration in eutrophic waters. To determine which combination of environmental
321 variables (explanatory variables) was represented by the dbRDA axes, multiple partial
322 correlations of each variable with each dbRDA axis was examined (Fig. 3). The first axis was
323 mainly defined by negative multiple partial correlations of both variables, NO_3 (r=-0.73) and flow
324 (r=-0.69), while the second axis was negatively related to NO_3 (r=0.69) and positively related to
325 flow (r=0.73). Pearson's correlations of eukaryotic species with each of the dbRDA axes were
326 examined to study the influence of the environmental changes on the community structure.
327 These results are summarized in Fig. 4. It was observed that the increase in flow as well as the
328 nitrate concentration were associated with the higher abundance of the genus *Acrostichus* sp.
329 and the nematode genera *Tobrilus* sp., *Koerneria* sp., and *Eumonhystera* sp. Correlations
330 between the presence of nitrate and nematoda in water has been previously studied (Song et
331 al., 2016). These results indicate a greater resistance of these eukaryotic genera to the UV dose
332 when the flow and nitrate concentration are increased.

333 **3.4. Eukaryotic organisms of public health concern**

334 Some eukaryotic microorganisms which could be present in drinking water microbiome such as
335 FLA, *Cryptosporidium*, some nematodes or fungi, pose a potential human health risk either
336 because of their intrinsic pathogenicity or because their ability to act as bacterial pathogen
337 carriers. These eukaryotic organisms are frequently unnoticed because their presence is not
338 monitored as a microbiological drinking water quality parameter.

339 In this sense, sequences from the genera *Blastocystis*, *Entamoeba*, *Acanthamoeba*,
340 *Hartmannella*, *Naegleria*, *Vanella*, *Microsporidium*, *Rhabditis*, *Tetrahymena*, *Paramecium*,
341 *Philodina*, *Daphnia* or *Caenorhabditis*, among others, have been identified in this study in the
342 drinking water microbiome even after UV disinfection (Fig. 5).

343 *Blastocystis* is an emerging pathogen in terms of its association with disease and zoonotic
344 potential (Thompson and Smith, 2011). Although sequences of *Blastocystis* genus have been
345 identified in the present study at low levels in UV treated samples, the human opportunistic
346 pathogenic species *Blastocystis hominis* has been only detected before UV disinfection.
347 Although, as shown in this work, this pathogen seems to be sensitive to UV, its sensitivity to
348 disinfection and the role of drinking water as transmission vehicle have not been well stablished
349 yet. *Entamoeba histolytica* is the causative agent of amoebiasis. It is undoubtedly of outmost
350 clinical significance since it results in ~100,000 human deaths annually (Nakada-Tsuki and
351 Nozaki, 2016). Despite the similarity between *E. histolytica* and *E. dispar*, the pathogenicity of
352 the latter is not clear and its role as a pathogenic bacteria carrier has been only demonstrated
353 for *E. histolytica* (Oliveira et al., 2015). Moreover, it is known that some FLA such as
354 A*canthamoeba* spp., *Naegleria fowleri* and *Balamuthia mandrillaris* are ubiquitous protozoa
355 that may behave as parasites under certain conditions, thus causing infections in humans and
356 leading to severe pathologies (Baldursson and Karanis, 2011). Sometimes, these FLA and other
357 non-pathogenic FLA, such as *Hartmannella or Vanella* can also bear pathogenic bacteria

358 (Delafont et al., 2013) or even *Cryptosporidium* oocysts (Scheid and Schwarzenberger 2011)
359 posing an additional risk. Several authors reported the presence of FLA in DWTPs and their ability
360 to survive to chlorine disinfection, thus reaching the final effluents and therefore water for
361 human consumption (Thomas et al., 2008; Corsaro et al., 2010; Moreno-Mesonero et al., 2017).
362 The identification of FLA after UV treatment, although their viability has not been proven,
363 implies that the UV dose should be revised since some FLA, such as *Acanthamoeba* or
364 *Hartmannella,* have been reported to resist UV doses of up to 990 J/m^2 (Cervero-Aragó et al.,
365 2014). Some FLA genera can grow and colonize DWTPs. Thus, although their concentration in
366 raw water is low, they can be present in high concentrations in the final water treatment steps
367 (Thomas and Ashbolt, 2011). Besides FLA, other non-pathogenic protozoa found in this work,
368 such as *Tetrahymena* and *Paramecium,* have been identified as bacterial carriers (Bichai et al.,
369 2008). Moreover, few studies have reported that members of zooplankton such as *Dhapnia* or
370 *Phylodinia* are also possible hosts of pathogenic microorganisms (Bichai et al., 2008; Callens et
371 al., 2018).

372 The nematode *Caenorhabditis,* identified in this work in both microbial communities before and
373 after the UV treatment, is a free living, multicellular invertebrate and resistant to disinfection
374 treatments. It has been previously reported that the species *Caenorhabditis elegans* is not a
375 human pathogen. However, it could be a natural host to some pathogenic microorganisms
376 (Cladwell et al., 2003; Zhang and Hou, 2013), thus acting as vehicle of its transmission through
377 water. The free-living nematode *Rhabditis*, presents both features, it is a human pathogen and
378 a bacterial carrier and can be attached to the biofilms formed in DWTPs. Although free-living
379 nematodes are not frequently associated to causing health threat, their presence represents a
380 water quality problem for the consumer (WHO, 2004).

381 *Microsporidium* is a unicellular fungus and an obligate intracellular pathogen. It has been found
382 in wastewater treatment plants (WWTPs) and even in DWTPs (Izquierdo et al., 2011) but, to our

383 knowledge, this is the first time that it was identified as part of a UV disinfected drinking water
384 microbiome.

385 Currently, scarce information about UV treatment to remove eukaryotic pathogens at DWTPs is
386 available. There are only few studies about specific groups of microorganisms, such as fungi (Ali
387 et al., 2017). However, human health concerning eukaryotes have proved to be resistant to
388 disinfection and they have been found at final products of DWTPs even after chlorine treatment
389 (Lin et al., 2014; Inkinen et al.,2019). Although the presence of pathogenic eukaryotes in the UV
390 drinking water microbiome has been showed in this report, further studies would be required
391 to better understand the real human health risk by stablishing their viability.

392 **3.5. Identification of potential bacterial pathogens associated to eukaryotic organisms.**

393 It is well known the role of some eukaryotic organisms as protectors or transmission vehicles of
394 bacteria in drinking water systems by harboring bacteria inside them or even carrying bacteria
395 attached to their surface. Therefore, in this study, the bacterial sequences of 18 aleatory
396 samples were also analyzed by 16S rRNA amplicon-based Illumina sequencing to perform a
397 preliminary study of these eukaryotic-bacteria possible associations in the DWTP ecosystem.
398 Although the samples were concentrated by a method to recover the eukaryotic organisms, it is
399 assumed that some bacteria bigger than 1µm could be retained by the filter. However, most of
400 the human pathogenic genus of bacteria found in the current study are smaller than 1µm in
401 width (*Pseudomonas* 0.5-0.8 µm (Iglewski BH. 1996); *Arcobacter* 0.2-0.9 µm (Pèrez-Cataluña et
402 al., 2018), *Aeromonas* 0.3 to 1.0 µm; *Legionella* 0.3-0.9 µm; *Parachlamydia* 0.25-0.3;
403 *Mycobacterium* 0.5-1 µm) (Boone et al., 2001), and that is why they should pass the filter and,
404 therefore, only be detected if they are somehow associated to eukaryote organisms. In fact,
405 other authors have previously reported that pathogenic bacterial species such as *Pseudomonas*
406 *aeruginosa* , *Staphylococcus* or *Kebsiella* or even bacteria higher than 5 µm length can pass even
407 through 0.45 µm filters (Hasegawa et al., 2003; Liu et al., 2019).

408 Moreover, the probability that the identification of the bacterial sequences could be due to
409 extracellular bacterial DNA (eDNA) suspended in the sample is very low mainly after UV
410 treatment because of the rapid damage that UV radiation produces on DNA (Gršković et al.,
411 2013).

412 A total of 1,334,513 bacterial sequences were recovered after amplicon sequencing. After
413 quality filtering and chimeras screening, 1,218,806 sequences remained. Samples were rarefied
414 to 37,420 sequences/sample to make comparisons among them in an equal basis. Thereafter,
415 sequences were clustered into 6,770 OTUs.

416 Overall, the most abundant bacterial phyla were Proteobacteria, Planctomycetes, Elusimicrobia
417 and Cyanobacteria, representing 80.59% of the total bacterial microbiota (Fig. 6).

418 Along the treatment process, the relative abundances of Alphaproteobacteria and
419 Betaproteobacteria decreased, and the opposite occurred for the class Gammaproteobacteria,
420 which contains many pathogenic genera, in finished water. In fact, the most abundant genera
421 identified belonged to Gammaproteobacteria class (Fig. 7).

422 A nMDS analysis was conducted to determine the overall relationships of bacterial genus among
423 samples and to explore in more detail the impact of UV treatment on the microbiome genus
424 composition. Genera did not cluster according to the treatment variable. Therefore, no
425 significant correlation was observed between UV treatment and the microbial community
426 composition (data not shown).

427 Several genera of concern to human health were identified among the bacterial sequences
428 recovered from the samples (Fig. 8). The most abundant were *Pseudomonas, Legionella,*
429 *Mycobacterium, Bacillus, Arcobacter* and *Aeromonas* . Among these genera, several species
430 have been identified as human pathogens. The 16S rRNA hypervariable region V3-V4, used in
431 this study, is widely used for bacterial communities' studies, however, sometimes it does not
432 have enough sequence diversity to distinguish at species level. The genus *Pseudomonas*

433 comprises more than 25 pathogenic species including *P. aeruginosa, P. syringae, P maltophilia*
434 and *P. fluorescens* (Iglewski BH, 1996). The sequences recovered, showed a match with *P.*
435 *aeruginosa* 16S rRNA sequence higher than 97%. *Legionella pneumophilla, P. aeruginosa* and
436 Non-Tuberculous Mycobacteria (NTM) such as *M. avium* have been described as an
437 opportunistic bacterium frequently found in drinking water systems (Lu et al., 2016). One of the
438 *Legionella* OTUs presented a 100% homology with the pathogenic specie *L. feelei*. Other OTUs
439 have been only identified at genus level. *Mycobacterium* OTUs have been only identified at
440 genus level, however, the sequences presented more than 97% homology with several species
441 including those pathogenic. Besides *B. cereus* and *B. subtillis*, other species such as *B. brevis*, *B.*
442 *cereus*, *B. circulans*, *B. lentus*, *B. licheniformis*, *B. mycoides*, *B. subtilis* and *B. thuringiensis* have
443 been reported to be toxic (Blackburn and McClure, 2009). Several species of *Aeromonas, and*
444 *Arcobacter* such as *Aeromonas sobria, Aeromonas caviae, Aeromonas veronii and Aeromonas*
445 *salmonicida, Arcobacter butzlerii, Arcobacter cryaerophillus and Arcobacter skirrowii* have been
446 recognized as human pathogens (Gugliandolo et al., 2007). The sequences recovered form
447 *Arcobacter* showed a >97% homology with some pathogenic species such as *A. suis*. The species
448 of *Aeromonas* known to cause human infection found in contaminated water are *A hydrophilla*,
449 *A. caviae*, *A. veronii A. dhakensis and A. sobria* (Figueras and Ashbolt, 2019). The last one, was
450 identified among the *Pseudomonas* sequences. It was observed that the frequency of most of
451 these genera was lower after UV treatment. However, in some samples the relative abundances
452 of some pathogens such as *Pseudomonas*, *Arcobacter* or *Aeromonas* even increased after UV
453 treatment. Some human waterborne pathogens have been reported to be carried and kept safe
454 from the disinfection treatments inside FLA, nematodes and even flagellate organisms (Samba-
455 Louaka, 2018; Moreno-Mesonero et al., 2019; Bichai et al., 2008). Therefore, in the same way,
456 the waterborne pathogens present in the analysed samples could be protected against
457 disinfection treatments by higher organisms resistant to the applied UV dose. In this work the
458 relationship between the identified bacterial carriers and/or potential pathogenic eukaryotes

459 and the potential bacterial human pathogens has been stablished. To our knowledge, this is the
460 first work in which this relationship has been studied in a DWTP with a UV disinfection
461 treatment. Although other authors have studied the variation of both eukaryotic and bacterial
462 communities in DWTPs before and after disinfection with chlorine, they have not stablished a
463 possible relationship between both communities (Dai et al., 2020).

464 The DistLM test established that 44.60% of the variation in the bacterial pathogenic community
465 was attributed to four eukaryotic carriers, finding a significant correlation with *Caenorhabditis*
466 (Table S6). The results of the sequential test showed that *Caenorhabditis c*ontributed with the
467 highest percentage of explained variance (p=0.05, 20.04%), followed by *Hartmannella* (p=0.107,
468 10.76%), *Daphnia* (p=0.221, 6.56%) and *Naegleria* (p=0,191, 7.23%). The contribution of each of
469 the eukaryotic carrier (explanatory variables) was represented by the dbRDA axes plot. (Fig. 9).
470 Pearson´s correlations of bacterial pathogens with each of the dbRDA axes were examined and
471 the correlation network were summarized in a network diagram in Fig. 10. It illustrates those
472 five of the ten pathogens showed positive and negative correlation with the carriers.
473 *Caenorhabditis* was associated with *Arcobacter* (r=0.68 in dbRDA1 and r=0.53 in dbRDA2) and
474 *Mycobacterium* (r=0.48 in dbRDA1). Several reports described the role of some nematodes as
475 potential transmission vehicles of bacterial pathogens which they have previously ingested,
476 (Bichai et al., 2008). Moreover, Zhang and Hou (2013) also reported that members of
477 *Caenorhabditis* are natural hosts of some bacteria, particularly, they showed that *C. elegans*
478 microbiota such as *Bacillus* and *Pseudomonas* can enhance the pathogenic resistance of the host
479 in different ways. Then, the fact that nematodes could survive to the conventional treatments
480 of water, as it has been described in this work, could pose a potential health risk due to their
481 role as bacterial carriers and protectors of pathogenic bacteria.

482 Moreover, a relationship between *Naegleria* and *Hartmannella* with the pathogenic bacteria
483 *Parachlamydia (*r=0.48 in dbRDA3) and *Arcobacter* (r=0.53 in dbRDA2) was also observed after
484 analysis. Both bacteria, *Parachlamydia* and *Arcobacter,* have been previously reported to be able

485 to resist fagocytation or even multiply inside FLA (Horn, 2008; Scheid, 2018). Particularly,
486 *Parachlamydia acanthamoebae,* the only specie of the genus, is defined as an endosymbiont of
487 FLA, which can also act as a reservoir of this human pathogenic specie (Horn et al., 2008). The
488 pathogenic ability of the species *Parachlamydia acanthamoebae* has been previously reported
489 (Greub and Raoult, 2002).

490 Despite our results of occurrence of some pathogenic bacteria after UV treatment could not be
491 robustly associated with the presence of some of the identified eukaryotic carrier organisms,
492 their presence could only be explained either because they were inside the eukaryotes, attached
493 to them or even attached to an unspecific non-filtered particle. However, in view of the
494 preliminary results obtained, further studies with other techniques such as microscopy or
495 Fluorescent *in situ* hybridization, used by other authors to elucidate bacteria-eukaryote
496 associations (Lacharme-Lora et al., 2009; Fu and Liu, 2019), should be carried out to confirm
497 symbiosis or parasitism particular cases.

498 Furthermore, because of the lack of specific information about UV resistance of bacterial
499 pathogens inside eukaryotic organisms, such as FLA or nematodes, among others, more research
500 is required to address this issue of concern. However, the microbiome characterization such as
501 this carried out in this study could influence future disinfection strategies of DWTPs. This may
502 ensure a better water quality and safety, thus preventing that some bacteria of concern to
503 human health that are not affected by the UV treatment (because they are protected by
504 resistant eukaryotic organisms), reach consumers.

505 **4. Conclusions**

506 In this study, the eukaryotic microbiome of a DWTP and the effect of the UV disinfection step of
507 the final product on the microbiome shape have been characterized for the first time. The health
508 concern eukaryotic genera *Blastocystis*, *Entamoeba*, *Acanthamoeba*, *Hartmannella*, *Naegleria*,
509 *Vanella*, *Microsporidium*, *Rhabditis*, *Tetrahymena*, *Paramecium*, or *Caenorhabditis*, among

510 others, have been identified in water samples after active carbon treatment and even after UV
511 disinfection treatment. Furthermore, the occurrence of bacterial human pathogens such as
512 *Mycobacterium, Pseudomonas* or *Arcobacter* in the DWTP samples has been associated with the
513 presence of high-level eukaryotic organisms which could act as bacterial carriers, thus being able
514 to protect bacteria from UV treatment. Due to the lack of information about the role of
515 eukaryotic organisms as transmission vehicles for bacterial pathogens in DWTPs with UV
516 disinfection, and the impact of our results on public health issues, more research should be
517 carried out to address this concern. Particularly, bacteria- eukaryote correlations elucidated in
518 this work should be supported by further specific studies that also include techniques such as
519 microscopy or Fluorescent *in situ* hybridization to confirm the symbiosis or parasitism
520 phenomena.

521

522

523 **Acknowledgements**

524 The authors are very grateful to the Town Hall of Valencia for their support to EMIVASA and "LA
525 PRESA" laboratory.

526 This research did not receive any specific grant from funding agencies in the public, commercial,
527 or not-for-profit sectors.

528

529 **Data availability statement**

530 Raw data can be found in zenodo under de DOI number 10.5281/zenodo.4607309

531

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Figure legends:

Fig. 1. Non-metric multidimensional scaling (nMDS) plot of eukaryotic communities (generated from the Bray–Curtis similarity matrix after square-root transformation of abundance) according to the ‘treatment stage’ (active carbon and ultraviolet) factor at phylum level (A) and at genus level (B).

Fig. 2 Relative abundances (%) of the eukaryotic phyla along the samples, according to the treatment stage (C: active carbon, UV: ultraviolet).

Fig. 3. dbRDA plot representing the model of variation in eukaryotic species community and its relationships to environmental variables. The length and direction of the vectors represent the strength and direction of the relationship.

Fig. 4. Metscape network analysis showing the relationship between eukaryotic species and environmental variables. The yellow nodes represent the axes of the dbRDA, defined by environmental variables and the white nodes represent eukaryotic species. The red lines indicate a positive correlation ($r>0.5$), while the blue lines indicate a negative correlation ($r>-0,5$).

Fig. 5. Heatmap representing the relative abundances (square root transformation of the percentage) of eukaryotic potential pathogens and bacterial carriers along the samples, according to the treatment stage.

Fig. 6. Relative abundances (%) of the bacterial phyla (A) and classes (B) associated to protozoa, according to the treatment stage (C: active carbon, UV: ultraviolet)

Fig. 7. Heatmap showing the relative abundances (square root transformation of the percentage) of bacterial Phyla/Class associated to the eukaryotic organisms along the samples, according to the treatment stage.

801 Fig. 8. Heatmap showing the main waterborne bacterial genus (square root transformation of
802 the relative abundance percentage) present in the samples, according to the treatment stage.

803 Fig. 9. dbRDA plot representing the model of variation in bacterial pathogens structure and their
804 relationships to eukaryotic carriers. The length and direction of the vectors represent the
805 strength and direction of the relationship. The first axis was mainly defined by *Caenorhabditis*
806 (r=0.75), while the second axis was related to *Hartmannella* (r=0.61) and *Caenorhabditis*
807 (r=0.65). Finally, the third and fourth axis were defined by *Naegleria* (r=0.91) and *Daphnia*
808 (r=0.86), respectively.

809 Fig. 10. Metscape network analysis showing the associated bacterial pathogens to eukaryotic
810 carriers. The yellow nodes represent the axes of the dbRDA, defined by eukaryotic carriers and
811 the white nodes represent bacterial pathogens. The red lines indicate a positive correlation
812 (r>0.5), while the blue lines indicate a negative correlation (r>-0,5).

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