

Salinity is the major driver of the global eukaryotic community structure in fish-canning wastewater treatment plants

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1 **Title**

2 Salinity is the major driver of the global eukaryotic community structure in fish-canning
3 wastewater treatment plants

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12 **Key Words:** *Eukarya* community, Eukaryotic diversity, NaCl concentration, Fish-canning WWTP,
13 Illumina sequencing

14

15 **Highlights**

16 NaCl concentration was a main driver in the modulation of the eukaryotic diversity

17 A greater eukaryotic diversity seems required for saline wastewater treatment

18 A high relative abundance of dark matter fungi was found, which was related to NaCl

19 *Ocystis (Chlorophyta)* is proposed as an eukaryotic bioindicator in saline WWTPs

20

21

22 **Abstract**

23 Fish-canning wastewater is characterized frequently by a high content of salt (NaCl), making
24 its treatment particularly difficult; however, the knowledge of the effect of NaCl on eukaryotic
25 communities is very limited. In the present study, the global diversity of eukaryotes in activated
26 sludges (AS) from 4 different wastewater treatment plants (WWTPs) treating fish-canning
27 effluents varying in salinity (0.47, 1.36, 1.72 and 12.76 g NaCl/L) was determined by sequencing
28 partial 18S rRNA genes using Illumina MiSeq. A greater diversity than previously reported was

29 observed in the AS community, which comprised 37 and 330 phylum-like and genera-like groups,
30 respectively. In this sense, the more abundant genus-like groups (average relative abundance
31 (RA) >5%) were *Adineta* (6.80%), *Lecane* (16.80%), *Dictyostelium* (7.36%), *Unclassified_Fungi7*
32 (6.94%), *Procryptobia* (5.13) and *Oocystis* (5.07%). The eukaryotic communities shared a
33 common core of 25 phylum-like clades (95% of total sequences); therefore, a narrow selection
34 of the eukaryotic populations was found, despite the differences in the abiotic characteristics of
35 fish-canning effluents and reactor operational conditions inflicted. The differences in NaCl
36 concentration were the main factor that influenced the structure of the eukaryotic community,
37 modulating the RAs of the different phylum-like clades of the common core. Higher levels of salt
38 increased the RAs of *Ascomycota*, *Chlorophyta*, *Choanoflagellata*, *Cryptophyta*, *Mollusca*,
39 *Nematoda*, Other Protists and Unclassified Fungi. Among the different eukaryotic genera here
40 found, the RA of *Oocystis* (*Chlorophyta*) was intimately correlated to increasing NaCl
41 concentrations and it is proposed as a bioindicator of the global eukaryotic community of fish-
42 canning WWTPs.

43

44 **Abbreviations**

45 AS: Activated sludge

46 CAS: conventional activate sludge

47 NMS: nonmetric multidimensional scaling

48 OTU: Operational taxonomic unit

49 PCA: Principal component analysis

50 RA: Relative abundance

51 TOC: Total organic carbon

52 TSS: Total suspended solids

53 VFA: Volatile fatty acids

54 VSS: Volatile suspended solids

55 WW: Wastewater

56 WWTP: Wastewater treatment plants

57

58 **1. Introduction**

59 World population growth has corresponded with an increase in the global consumption per
60 capita of fish and seafood, which has doubled since 1973 with >3% average annual increase
61 (FAO, 2018). Nowadays, fish and seafood represent about 17% of the world's animal protein
62 intake (Laso *et al.*, 2018). The proportion of fish processed into prepared or preserved forms
63 equals 10% of total human fish consumption, amounting >15 million tonnes (live weight) per
64 year globally. Among the different preservation methods, canning transformation is of great
65 importance, as it improves shelf life enabling storage for several years. In the Iberian Peninsula,
66 the canning sector is the main segment of fishing manufacturing industry with >85 factories,
67 located mainly in the coastal areas (Val del Rio *et al.*, 2018).

68 The fish canning industry consumes large amounts of water for cleaning and washing raw
69 materials, boiling and cooking processes, resulting in equally large quantities of wastewater
70 (WW) (Lim *et al.*, 2003). Fish canning effluents require treatment before their disposal to prevent
71 environmental damage, but they are characterized generally by a high content of salt and
72 organic matter (including oil and grease), which makes their treatment particularly difficult (Val
73 del Rio *et al.*, 2018). These processes are performed in wastewater treatment plants (WWTPs),
74 which usually include primary units aimed to remove particulate matter and oils by
75 physicochemical treatments, and in some cases secondary units that normally consist of
76 biological reactors for the removal of dissolved matter (Pham *et al.*, 2020).

77 In secondary treatments, Conventional Activated Sludge (CAS) systems are robust and
78 widely employed to treat both urban and industrial wastewater because of their versatility.
79 Besides, the combination of aerobic and anoxic periods or basins promotes the removal of
80 nutrients and organic matter together in the same system. Nevertheless, this WWTP approach

81 includes only treatment, and no valorisation is performed. For systems including anaerobic
82 digestion, organic matter is converted into biogas. However, nutrients are not removed in the
83 valorisation unit (the anaerobic digester), so an extra unit is necessary (normally, a CAS or an
84 anammox unit) where nutrients and/or the remaining organic matter are removed. Moreover,
85 high salinity is known to inhibit methanogenesis, lowering biogas yield (Palmeiro-Sánchez *et al.*,
86 2013). Therefore, the use of this type of treatment can be challenging in high-salinity systems as
87 fish-canning WWTP. Novel wastewater treatment technologies include the use of biofilms or
88 membranes. Although the results obtained are promising (Carrera *et al.*, 2019), the full-scale
89 application of these technologies is still scarce, and most of the industrial fish-canning WWTPs
90 operate by using a CAS or an anaerobic digester.

91 The biological processes taking place in the secondary treatments are based on the activity
92 of a consortium of microbial populations. Among the different microorganisms, eukaryotes are
93 pivotal players in WWTP bioreactors (dos Santos *et al.*, 2014; Hirakata *et al.*, 2019). In this sense,
94 some genera have been described as beneficial in pollutant removal, such as members of the
95 *Fungi*, *Chlorophyta*, or Rhizaria (an unranked group), which contribute to reduce nitrogen and
96 phosphorus concentrations in WW (Zahedi *et al.*, 2019). Also, phyla *Ciliophora*, *Rotifera* and
97 *Nematoda*, or Stramenopiles (unranked group), are bacterivores that regulate the proliferation
98 of bacterial populations (Šimek *et al.*, 2019). However, some eukaryotic genera have also been
99 reported as harmful for technical or health management reasons (Zahedi *et al.*, 2019), pointing
100 out the importance of microbial communities' identification.

101 High-throughput sequencing analysis of 18S rRNA gene has revolutionised the
102 understanding of eukaryotes diversity and abundance in WWTPs (Cooper *et al.*, 2016). Recently,
103 some efforts have been made to characterize specific branches of the tree of life of the *Eukarya*
104 domain by using these tools (Hirakata *et al.*, 2019; Liu *et al.*, 2019). Despite these attempts, the
105 knowledge of the biodiversity of different eukaryotic communities in WWTP is still very limited
106 and unidentified eukaryotes with important roles may be overlooked.

107 Different physicochemical parameters affect the eukaryotic communities in WWTPs, among
108 which salinity might be a key factor for their composition and robustness (Horikoshi *et al.*, 2010).
109 However, little is known about the effect of NaCl in these communities in fish-canning WWTPs.
110 As effluents coming from fish-canning industries are characterized by variable salinity
111 concentrations (2-35 g NaCl/L, Cristóvão *et al.*, 2016), it is essential to comprehensively
112 characterize their global eukaryotic communities and to establish how salinity modulates the
113 dynamics of the populations that comprise them.

114 In order to go deeper into the existing relationships among the different eukaryotic clades,
115 and aiming to establish how operational parameters and WW influent characteristics drive the
116 biodiversity of those communities, several goals are expected to be fulfilled in the present work:
117 (1) to determine the global eukaryotic biodiversity in fish-canning WWTP bioreactors; (2) to
118 investigate the relationships among the different eukaryotic communities; (3) to examine the
119 effect that different physicochemical parameters, mainly salinity, produce in the composition of
120 the *Eukarya* community; and (4) to propose bioindicator genera of eukaryotes whose abundance
121 correlates to the shifts of different operational parameters in fish-canning WWTPs.

122 The answers to these objectives will expand the knowledge of the diversity of the eukaryotic
123 communities in the biological treatment of high-salinity effluents, helping to infer their potential
124 roles. These results will be also relevant for the biological treatment of other saline effluents,
125 such as those derived from chemical, pharmaceutical, agricultural, or aquacultural industries
126 (Zhao *et al.* 2020).

127

128 **2. Experimental procedures**

129 *2.1 Description of the WWTPs source of biomass samples*

130 Duplicate sets of samples (n=2), referred to as F1 to F4, were taken in four different WWTPs
131 of fish-canning industries in Galicia (Northwest of Spain) under steady-state conditions. F1 and
132 F2 were CAS systems operating at low (0.47g NaCl/L) and moderate (1.72 g NaCl/L) salinities,

133 respectively, while F3 (moderate salinity, 1.36 g NaCl/L) and F4 (high salinity level, 12.76 g
134 NaCl/L) valorise organic matter by converting it into biogas in anaerobic units before using CAS
135 for nutrient removal (Fig. 1).

136 *2.2 Chemical determinations*

137 Collected liquid samples were characterized as elsewhere described (Val del Rio *et al.*, 2018;
138 Pedrouso *et al.*, 2021). The pH value was measured with a glass electrode (Crisson GLP22). Total
139 Suspended Solids (TSS) and Volatile Suspended Solids (VSS) were analysed, in bulk samples,
140 according to the Standard Methods (APHA, 2005). Samples were filtered using a cellulose-ester
141 filter (Advantec, Japan) when soluble parameters were determined. The concentrations of
142 sodium (Na⁺) and chloride (Cl⁻) were analysed by ion chromatography (Metrohm 861,
143 Switzerland). Volatile fatty acids (VFA) concentrations were measured by gas chromatography
144 (6850 Series II, Agilent Technologies). Total organic carbon (TOC) concentration was determined
145 by a Shimadzu analyser (TOC-L, automatic sample injector Shimadzu ASI-L). Ammonia was
146 measured spectrophotometrically at 640 nm (Bower and Holm-Hansen, 1980).

147 *2.3 DNA isolation*

148 Different volumes of activated sludge (AS) samples (9 - 13.5 mL) were centrifuged at 14,000
149 rpm at 22 °C for 1 min, the supernatants were discarded and biomass kept at -20 °C until use.
150 Two independent biological replicates were used for each DNA extraction, using the FastDNA
151 Spin kit for Soil in the FastPrep-24 instrument (MP Biomedicals, Irvine, CA, USA) according to the
152 manufacturer's instructions, and stored at -20 °C until further use.

153 *2.4 Next-generation sequencing library preparation*

154 DNA extracts were provided to RTL Genomics (Lubbock, Texas, USA) for partial 18S rRNA
155 Illumina MiSeq sequencing. PCR amplification products of the 18S rRNA V9 region gene were
156 generated using the Earth Microbiome Project primers Euk1391f (5'-GTACACACCGCCCGTC-3')
157 (Lane, 1991) and EukBr (5'-TGATCCTTCTGCAGGTTACCTAC-3') (Medlin *et al.* 1988). As the
158 forward primer is a highly-conserved three domain-level primer, there is a risk of recovery of

159 non-eukaryotic tags even when used in combination with an eukaryote-specific PCR primer
160 (Cooper *et al.*, 2016). However, the highly conserved nature of the eukaryote-specific PCR
161 primer Euk1391f was reported to recover higher diversity, compared to other alternative
162 primers designed specifically for eukaryotes amplification only (Amaral-Zettler *et al.*, 2009).

163 *2.5 Bioinformatic analysis of 18S rRNA gene amplicon libraries*

164 Raw Illumina sequencing data of industrial WWTPs were analysed using the Mothur v.1.42.3
165 pipeline (Schloss *et al.*, 2009). Full details of the followed procedures are provided in
166 Supplementary material, Section 2.

167 *2.6 Sequencing coverage estimation and α -diversity indices*

168 Good's coverage index and Simpson, Shannon and Chao-1 indices were calculated according
169 to Esty (1986) and Hill *et al.* (2003), respectively.

170 *2.7 Statistical analyses*

171 Analyses of variance of the different parameters among the samples were made using the
172 non-parametric Kruskal-Wallis and Conover-Iman combined tests, with a 95% significance level
173 ($p < 0.05$) using the package XLSTAT v2019.3.2 (Addinsoft, New York, USA). Heatmaps were
174 constructed using the average clustering method, and generated with R studio v.3.4.1 package
175 (Rstudio, Boston, MA, USA). Principal component analysis (PCA) and nonmetric
176 multidimensional scaling (NMS) ordination analysis were constructed by using PC-ORD software
177 (Wild Blueberry Media, Oregon, USA). Full details are described in Supplementary materials.
178 Additionally, correlations analyses of each one of the abiotic variables and population RAs at the
179 genus-like level were made using Spearman's rank correlation coefficients, using the XLSTAT
180 software.

181 *2.8 Nucleotide sequence accession numbers*

182 The nucleotide sequences reported in this work have been deposited in GeneBank under
183 the accession number SUB7560150.

184

185 3. Results

186 3.1 Wastewater and sludge characterization

187 WW and AS samples were characterized in order to determine the different conditions to
188 which microorganisms were exposed. The results displayed in Table 1 show the high variability
189 of fish-canning effluents. As previously stated, salinity was low in F1, moderate in F2 and F3, and
190 high for F4. F4 is a high-load system, with a TOC concentration in the influent of approximately
191 2 g/L, while the remaining facilities treat low or moderate organic loads (0.26-0.49 g/L).

192 The PCA plot of the physico-chemical properties explains 87% of the total variability among
193 the 4 WWTPs (Fig. S1). The samples were distributed around all the 2D-space created, indicating
194 a high variability of the different abiotic parameters. NaCl concentration in WW was the main
195 factor driving the ordination of the samples, as it was the variable more strongly related to the
196 principal component 1.

197 3.2 Next-generation sequencing library summary

198 Microbial community structures of AS retrieved from the four WWTPs were determined by
199 Illumina sequencing. Overall, a total of 164,128 high-quality sequences were obtained,
200 corresponding to 5,148 unique sequences. Posteriorly, sequences were *de novo* clustered into
201 3,070 operational taxonomic units (OTUs, 3% divergence). On average, 64.38% of the
202 representative sequences of each OTU were classified within the *Eukarya* domain, while 31.50%
203 were related to *Bacteria*, and 3.81% were not classified into any domain (Fig. 2). Unclassified
204 sequences and sequences classified as *Bacteria* were filtered and excluded, and 106,158
205 sequence reads were retained and used for subsequent analyses.

206 3.3 Overall eukaryotic community structure

207 The total number of eukaryotic OTUs was 1,413 (Table S1). In order to facilitate the analysis
208 of the composition and structure of the eukaryotic community, the different clades were
209 grouped according to their corresponding kingdom-like groups, as detailed in Supplementary
210 material (Section 2.1). The relative abundance (RA) of the kingdom-like groups was 27.74% for

211 Protista, 11.59% for *Fungi*, 51.42% for *Metazoa*, 5.44% for *Viridiplantae* and related, and 3.81%
212 for Other Eukaryotes (Fig. 3). There were statistically significant differences in the RAs of all
213 kingdom-like groups among the different AS samples.

214 At the phylum-like level, the 1,413 OTUs were classified into 37 different taxonomic groups
215 (27 different phyla and 10 groups unclassified at this level, Fig. 4 and Table S1). The 10 most
216 abundant groups (in decreasing order) were *Rotifera*, *Ciliophora*, an unranked group of
217 Amoebozoa, Unclassified_Fungi, *Euglenozoa*, *Chlorophyta*, *Nematoda*, *Zoopagomycota*,
218 *Heterolobosea* and *Apicomplexa*, that accounted for >95% of the overall eukaryotic community.
219 Generally considered, those 37 phylum-like groups had unequal distributions among the AS from
220 the four WWTPs (Fig. 4, Table S2).

221 The heatmap of the RAs of the different phylum-like taxa (Fig. 5) indicates a strong
222 assemblage of the eukaryotes in the different WWTPs, as the majority of the groups were
223 prevalent populations (and therefore were over-represented) in each specific facility. Briefly,
224 *Euglenozoa* and *Rotifera* dominated in F1, *Rotifera* in F2, Amoebozoa and *Ciliophora* in F3, and
225 *Chlorophyta* and unclassified *Fungi* in F4. The exceptions were Unclassified Alveolata,
226 *Arthropoda*, *Chordata*, and *Rodophyta*; that were more evenly distributed among the 4 samples.
227 The clustering of the different samples showed that F1 and F2 belonged to the same cluster,
228 followed in similarity by the F4 sample and, finally, F3 that was clustered apart in a specific
229 group.

230 As indicated by the Good's Coverage index (Table 2), the sequencing depths of the 8
231 amplicons of eukaryotic communities had an adequate level, whereas the values of observed
232 OTUs, Shannon and Chao-1 indices were not different among samples, and the Simpson index
233 was the highest in F2 and lowest in F3, with intermediate values for F1 and F4 (Table 2).

234 3.4 Community structure at the phylum- and genus-like levels

235 3.4.1 Protista community structure

236 The 507 protists OTUs were distributed (sorted in decreasing order) as *Ciliophora* (10.33%),
237 Amoebozoa (9.10%), *Euglenozoa* (6.10%), *Apicomplexa* (1.20%), Alveolata not classified (0.53%),
238 Other_Protists (0.35%) and *Cryptophyta* (0.12%) (Fig. S2a and Table S2).

239 Statistical differences of the RAs of the different phylum-like groups of Protista were found
240 among the 4 facilities analysed (Table S2). The dominant groups in the F1 samples were
241 *Euglenozoa* and *Ciliophora*; with average abundances of 39.93% and 10.69%, respectively (Fig.
242 S2a and Table S2). *Ciliophora* prevailed in F2 and F4 amplicon libraries (average RAs 5.07% and
243 7.60%, respectively), while in F3 samples the prevalent groups were Amoebozoa and *Ciliophora*,
244 with average RAs of 39.17% and 23.50%, respectively.

245 The 507 protists OTUs were affiliated to 93 different genus-like clades, of which only 25 had
246 RAs >0.1% (Table S1). The F1 and F2 samples were mainly dominated by *Procryptobia* (37.35%)
247 and *Opercularia* (3.65%), respectively (Fig. S2b). In contrast, samples F3 and F4 comprised a
248 wider spectrum of numerically prevalent genus-like clades. *Dictyostelium* (33.25%),
249 *Cochliopodium* (16.07%), *Hemolivia* (6.45%), *Opercularia* (4.72%) and *Vorticella* (2.81%) were
250 the more abundant in F3. F4 samples were dominated by *Pseudokeronopsis* (3.55%), *Nassula*
251 (3.06%), *Rhynchomonas* (2.33%) and *Carpediemonas* (1.40%).

252 3.4.2 Fungi community structure

253 A total of 196 OTUs were classified within the *Fungi*, which were taxonomically affiliated to
254 7 different phyla (sorted in decreasing RA order): *Zoopagomycota* (2.49%), *Blastocladiomycota*
255 (0.72%), *Ascomycota* (0.52%), *Microsporidia* (0.28%), *Chytridiomycota* (0.17%), *Basidiomycota*
256 (0.16%) and *Mucoromycota* (0.09%) (Fig. S3a and Table S2). In addition, 7,611 sequences (7.17%)
257 were not classified into any fungal phylum-like clade. There were little significant differences in
258 RAs of the different fungal phylum-like clades among samples (Table S2), compared to those
259 observed for the protist community. *Blastocladiomycota* (0.58%) and *Zoopagomycota* (10.95%)
260 were significantly more abundant in the F1 and F3 samples, respectively, while in F2 both
261 *Microsporidia* (0.58%) and *Blastocladiomycota* (0.56%) co-dominated. It is worth noting that the

262 F4 samples were characterized by a statistically higher abundance of sequences unclassified at
263 the phylum-like level (37.78%), compared to the other facilities (Fig. S3b and Table S2).

264 The 196 OTUs were classified into 81 different genus-like groups, 29 of which displayed a RA
265 >0.01% in the overall eukaryotic community. The F1 and F2 samples were characterized by the
266 presence of a broader number of dominant genus-like clades, compared to the other two
267 WWTPs (Fig. S3b). The main fungal genus-like clades in F1 samples were *Catenophlyctis* (2.18%),
268 *Piptocephalis* (0.38%), *Glomus* (0.25%), *Basidiobolus* (0.22%), *Galactomyces* (0.22%) and one
269 unclassified genus (Unclassified_Fungi9) (0.22%). *Mitosporidium* (0.58%), *Catenophlyctis*
270 (0.56%) and *Hyaloraphidium* (0.32%) dominated in the F2 amplicon libraries. Finally, the RAs of
271 *Acaulopage* and Unclassified_Fungi7 were higher-than-average in F3 (10.95%) and F4 (37.30%)
272 samples, respectively.

273 3.4.3 Metazoa community structure

274 The *Metazoa* community was composed of 334 different OTUs, distributed among 10
275 different phylum-like clades which were, in decreasing order of RA, *Rotifera* (47.27%),
276 *Nematoda* (3.00%), *Gastrotricha* (0.28%), *Cnidaria* (0.26%), *Arthropoda* (0.22%),
277 *Platyhelminthes* (0.12%), *Chordata* (0.01%), *Mollusca* (<0.01%) and *Annelida* (<0.01%) (Fig. S4a
278 and Table S2). The RAs of several phylum-like clades presented statistical differences among the
279 4 WWTPs (Table S2). There is important to note, that *Rotifera* were prevalent in the global
280 eukaryotic community (47.27%), and the most abundant phylum-like clade for F1 (36.17%), F2
281 (88.53%) and F3 samples (5.42%). Although *Rotifera* were also widely present in the F4 sample
282 (4.06%), *Nematoda* reached a higher RA (15.71%, Fig. S4a and Table S2).

283 The 334 OTUs were distributed into 39 different genus-like clades, 22 of which displayed
284 RAs >0.01% (Table S1). According to Fig. S4b, the samples with a lower level of salinity (F1, F2
285 and F3) were dominated by the same 3 genus-like groups of the *Rotifera*: *Adineta*, *Lecane* and
286 *Philodina*, whose RAs were 26.06%, 7.41% and 2.29% for F1, respectively; 48.57%, 33.92% and
287 5.26% for F2, respectively; and 4.52%, 0.58% and 0.28% for F3, respectively. On the other hand,

288 amplicon libraries from F4 samples were dominated by *Diplolaimella* (15.06%), and *Philodina*
289 (2.73%).

290 3.4.4 *Viridiplantae* and related community structure

291 The phyla *Chlorophyta* and *Streptophyta* belonging to the *Viridiplantae* kingdom, the
292 *Rhodophyta*, *Bacillariophyta* and the group of the Stramenopiles or heterokonts, displayed
293 average RAs of 5.29%, 0.14%, 0.81%, 0.07% and 0.01%, respectively (Fig. S5a). Those 5 taxa were
294 grouped into a single kingdom-like group named *Viridiplantae* and related, and included 202
295 different OTUs.

296 The RAs of those 5 phylum-like clades were statistically different among samples, with
297 exception of the *Bacillariophyta* (Table S2). The dominant phylum-like clade was *Chlorophyta*
298 (average values of 1.55%, 2.21%, 3.37% and 16.93% for F1, F2, F3 and F4 libraries, respectively)
299 (Fig. S5a and Table S2).

300 The 202 OTUs were classified into 60 different genus-like clades, 18 of which had RAs >0.01%
301 (Fig. S5b). F2, F3 and F4 samples were characterized by the same community structure for this
302 kingdom-like group. The planktonic genus *Oocystis* was mainly dominant, showing RAs of 0.90%,
303 2.19%, 3.00% and 16.71% in the F1, F2, F3 and F4 samples, respectively (Fig. S4b).

304 3.4.5 Other Eukaryotes' community structure

305 The sequences that could not be classified into a certain kingdom-like clade were
306 grouped together and named Other Eukaryotes. This miscellaneous group comprised 174 OTUs,
307 distributed (in decreasing order of RA) into the clades *Heterolobosea* (1.82%) (phylum level),
308 *Choanoflagellata* (class level) (0.70%), *Cercozoa* (0.26%) (phylum level), *Jakobida* (0.20%) (order
309 level), eukaryotic sequences not classified (Other_unclassified_eukaryotes, 0.17%), Rhizaria not
310 ascribed to a certain phylum (0.03%) and Aphelida (>0.01%) (Fig. S6a and Table S2).

311 The RAs of the different phylum-like clades were statistically different among the 4
312 WWTPs (Fig. S6a and Table S2). The dominant groups were *Heterolobosea* (7.62%) and
313 *Choanoflagellata* (3.23%) in the F3 and F4 samples, respectively.

314 The 174 OTUs distributed into 56 different genus-like clades, 18 of which had RAs
315 >0.01% (Table S1). The F1 and F2 samples were characterized by a more homogenous
316 distribution of genus-like clades, compared to the F3 and F4 samples (Fig. S6b). In the F1 and F2
317 samples, no dominant groups were found. However, *Naegleria* was over represented in F3
318 (7.48%), while *Salpingoeca* and *Monosiga* were dominant in the F4 samples (2.28% and 0.92%,
319 respectively).

320 3.5 Co-occurrence patterns inside the eukaryotic community structure

321 The heatmap of the genus-like clades of the 5 kingdom-like taxa are showed in Fig. S2c, 3c,
322 S3c, S4c and S5c. In general, the amplicon libraries of F1 and F2 samples clustered together for
323 each kingdom-like group except for the fungal communities, as in this case the samples with
324 higher level of similarity were F2 and F3. The dominance patterns indicated that the majority of
325 the genus-like clades were prevalent populations in a specific sample, except for fungal
326 communities, which had a lower number of prevalent populations.

327 Spearman correlations analyses were used to investigate the relationships among the 37
328 different phylum-like clades in the samples retrieved from the 4 WWTPs. The pairwise
329 correlations were ordered hierarchically into three different clusters (Fig. 6). There was a large
330 number of strong positive correlations, while many fewer negative correlations were found. A
331 total of 99 robust correlations were identified among the 37 phylum-like groups: 35 in cluster 1,
332 38 in cluster 2, and 23 in cluster 3. In each one of the 3 clusters there were a hot spot zone of
333 correlations, the “correlation island” composed by *Apicomplexa*, *Amoebozoa*, *Ciliophora*,
334 *Heterolobosea*, *Jakobida*, *Streptophyta* and *Zoopagomycota* in cluster 1, *Ascomycota*,
335 *Chlorophyta*, *Choanoflagellata*, *Cryptophyta*, *Mollusca*, *Nematoda*, *Other_Protists*, and
336 *Unclassified_Fungi* in cluster 2, and *Chytridiomycota*, *Gastrotricha*, *Microsporidia*, *Rhodophyta*,
337 *Rotifera* and *Unclassified_eukaryotes* in cluster 3.

338 In order to deeper analyse the interaction patterns, the correlations among RAs of the
339 genus-like clades of each one of the 3 clusters were also determined. Cluster 1 was composed

340 by members of phyla *Annelida*, *Apicomplexa*, *Bacillariophyta*, *Basidiomycota*, *Cercozoa*,
341 *Ciliophora*, *Heterolobosea*, *Porifera*, *Streptophyta* and *Zoopagomycota*, the order *Jakobida*, and
342 the unranked groups Amoebozoa and Aphelida. In this analysis, statistically significant relations
343 were found for all the 47 genus-like taxa included in this cluster, representing a co-occurrence
344 level of 19.52% (211 pairwise correlations, 1,081 possible, Fig. S7). Cluster 2 involved the phyla
345 *Arthropoda*, *Ascomycota*, *Chordata*, *Chlorophyta*, *Cnidaria*, *Cryptophyta*, *Mollusca* and
346 *Nematoda*, the order *Choanoflagellata* and the unranked groups Alveolata_unclassified,
347 Other_Prostists, Stramenopiles and Unclassified_Fungi. There were 480 relationships
348 representing 31.37% of the 1,540 potential co-occurrence patterns among the 56 genera
349 belonging to this cluster (Fig. S8). Finally, Cluster 3 was integrated by phylum-like clades
350 *Blastocladiomycota*, *Chytridiomycota*, *Euglenozoa*, *Gastrotricha*, *Microsporidia*, *Mucoromycota*,
351 *Platyhelminthes*, *Rhizaria*, *Rhodophyta* and *Rotifera*, and the unranked group
352 Unclassified_eukaryotes. The number of pairwise correlations among the 29 different genus-like
353 members of cluster 3 was 93, which represented 22.91% of the 406 set of possible co-occurrence
354 patterns (Fig. S9).

355 The phylum-like clades shared among all amplicon libraries were 25, comprising 95% of the
356 106,158 eukaryotic sequences. Similarly, 19 genus-like groups were common to the 4 WWTP
357 samples (68% of overall eukaryotic sequences). Therefore, there was a high level of synchrony
358 among samples. The shared phylum- and genus-like clades are listed in Supplementary material
359 Section 2.2.

360 3.6 Linking eukaryotic community structure to wastewater and sludge characteristics

361 A NMS analysis of the samples from the 4 WWTPs was carried out, based upon the RAs of
362 the 37 eukaryotic phylum-like taxa, relativized to the maximal taxon abundance for a specific
363 sample (Fig. 7). The two-coordinate axes explained 84% of the total variation, with a low 2D-
364 stress value (0.023). The 8 amplicon libraries from the 4 WWTPs were distributed differently in
365 the NMS space. F1 and F2 samples were more similar than F3 and F4 samples, which in turn

366 were placed apart independently. Analogous results of similitude among samples were observed
367 in the heatmap of the 37 phylum-like taxa (Fig. 5). *Blastocladiomycota*, *Chytridiomycota*,
368 *Cnidaria*, *Euglenozoa*, *Gastrotricha*, *Microsporidia*, *Mucoromycota*, *Platyhelminthes*, *Rhizaria*,
369 *Rhodophyta*, *Rotifera* and *Unclassified_eukaryotes*, were grouped around the F1 and F2 sample
370 scores. Similarly, *Annelida*, *Amoebozoa*, *Aphelida*, *Apicomplexa*, *Bacillariophyta*, *Basidiomycota*,
371 *Cercozoa*, *Ciliophora*, *Heterolobosea*, *Jakobida*, *Porifera*, *Streptophyta* and *Zoopagomycota*
372 clustered around the ordination of F3 samples. Finally, F4 amplicon libraries were mainly related
373 to *Alveolata*, *Arthropoda*, *Ascomycota*, *Choanoflagellata*, *Chordata*, *Chlorophyta*, *Cryptophyta*,
374 *Mollusca*, *Nematoda*, *Other_Prostists*, *Stramenopiles* and *Unclassified_Fungi*.

375 The vectors representing the physicochemical variables of the WW and sludges from the
376 different WWTPs were overlapped on the NMS ordination (Fig. 7). Pairwise Pearson product-
377 moment (r) correlations between the shifts of the different abiotic variables and the RAs of the
378 37 phylum-like clades shown in Fig. 7 are detailed in Table S3. Absolute values of $r > 0.7$ were
379 considered as strong correlations. The pH in WW and AS, TOC in WW, and NaCl concentration
380 in WW and AS, were positively related to *Alveolata*, *Arthropoda*, *Ascomycota*, *Chlorophyta*,
381 *Choanoflagellata*, *Chordata*, *Chytridiomycota*, *Cryptophyta*, *Mollusca*, *Nematoda*,
382 *Other_Prostists*, *Stramenopiles*, *Unclassified_eukaryotes* and *Unclassified_Fungi*. On the other
383 hand, NH_4^+ and VFA concentrations in WW, and VSS concentration in AS, were highly related to
384 *Amoebozoa*, *Annelida*, *Aphelida*, *Apicomplexa*, *Bacillariophyta*, *Basidiomycota*, *Cercozoa*,
385 *Ciliophora*, *Heterolobosea*, *Jakobida*, *Porifera*, *Streptophyta* and *Zoopagomycota*. In contrast,
386 the content of NH_4^+ in the WW was negatively related to *Alveolata*, *Arthropoda*, *Chordata*,
387 *Chytridiomycota*, *Cnidaria*, *Stramenopiles* and *Unclassified_eukaryotes*. Finally, the VFA
388 concentration in WW and VSS concentration in AS were negatively correlated to *Alveolata*,
389 *Arthropoda*, *Chordata*, *Chytridiomycota*, *Cnidaria*, *Mucoromycota* and *Unclassified_eukaryotes*.

390 3.7 Proposal of eukaryotic taxa as bioindicators in fish-canning WWTPs

391 Finally, pairwise Spearman's rank correlations between the abiotic variables and the RAs of
392 taxa at the genus-like level were calculated, and a high number of significant correlations were
393 found (Table S4). The ten strongest positive or negative correlations are displayed in Tables 3
394 (abiotic properties of WW) and 4 (abiotic properties of AS). pH in WW and NaCl content in both
395 WW and AS were highly correlated among them (Fig. S1); therefore, these variables correlated
396 similarly with the RAs of the same genus-like clades. Parallel trends were also observed for TOC
397 in WW and VSS concentration in AS, while the rest of the physico-chemical properties were
398 correlated to biotic data independently. Despite different genus-like clades were intimately
399 related to NaCl concentration, it is worth mentioning that the RA of *Oocystis* had the maximum
400 level of correlation with salinity in both WW and AS (Tables 3 and 4). This genus-like taxon may
401 play a pivotal role in the eukaryotic community of the analysed WWTPs, since it was within the
402 common core and reached high RAs in all the samples (0.9-20.0%); therefore, it is proposed as
403 a bioindicator of the response of eukaryotic microbial communities to the shifts of NaCl
404 concentration in fish-canning treatment systems.

405

406 **4. Discussion**

407 *4.1 Community structure at the kingdom-like level*

408 In this work, 259-329 eukaryotic OTUs were detected in AS samples (Table 2), with no
409 statistical differences of their richness among the four WWTPs. In contrast, a much lower
410 number of eukaryotic OTUs has previously been recorded in conventional WWTPs (García-Ruíz
411 *et al.*, 2020) and WWTPs treating sewage from chemical industries (Zhao *et al.*, 2019). Analysis
412 of the biodiversity at the phylum-like level allowed us to differentiate 37 groups (Table S2). In
413 contrast, Chouari *et al.* (2017) described 16 different phylum-like groups by cloning and
414 sequencing DNA isolated from different parts of an urban WWTP, while García-Ruiz *et al.* (2020)
415 reported only 19 phylum-like taxa when they analysed the biodiversity in the anaerobic digester
416 of a pilot-scale WWTP bioreactor. Therefore, compared to the previous observations in other

417 WWTPs, the results presented here revealed a greater diversity of the eukaryotic communities
418 in AS of fish-canning WWTP and, similarly, suggest that the treatment of the high-strength
419 and/or saline influents entering the fish-canning treatment facilities required a higher number
420 of eukaryotic groups than in other WWTPs.

421 4.2 Eukaryotic biodiversity synchrony

422 The synchrony of the eukaryotic biodiversity among the 4 WWTPs, determined as the
423 number of shared phylum-like clades present in each one of the different AS samples (Fig. 4 and
424 Table S3), was noteworthy. Therefore, the variations either in physic-chemical parameters or
425 the operational design of the WWTPs did not result in a drastic modification of the eukaryotic
426 diversity at the phylum-like level, but rather modulated the community structure (Fig. 4 and
427 Table S2). Besides, the OTUs richnesses and the values of the Shannon and Chao-1 indices were
428 not significantly different among samples (Table S2). In contrast, a lower level of synchrony has
429 been previously described in the eukaryotic composition of WWTPs by other authors. Zahedi *et al.*
430 *al.* (2019) reported that only 22% of sequences at the phylum-like level were shared in 4 rural
431 WWTPs. Similarly, Matsunaga *et al.* (2014) indicated that the abundance of shared eukaryotic
432 OTUs in sludges from different WWTPs was around 10%; however, in this work the common
433 core was composed by 34% of the identified OTUs. This suggests that the characteristics of fish-
434 canning influents produce a stronger selection of the eukaryotic communities than that
435 observed in other types of WWTPs.

436 The likenesses among the AS samples were also analysed globally taking into account the
437 RAs of taxa at the phylum and genus-like levels through construction of heatmaps. Considered
438 generally, there was a higher level of similarity between the F1 and F2 samples than for the
439 other two WWTPs (Fig. 4 and Figs. S2c, S3c, S4c, S5c and S6c), except for the fungal communities,
440 wherein the higher level of similarity was observed between F2 and F3, both bearing moderate
441 salinity. Taking into account that the F1 and F2 WWTPs have the same configuration, the use of
442 CAS systems resulted in a more specific selection of the eukaryotic community than that

443 observed for F3 and F4 facilities. Besides, the NMS ordination based on the RA of the 37 phylum-
444 like clades confirmed this result (Fig. 7). Therefore, the type of configuration influenced the
445 development of the eukaryotic community structures of the 4 WWTPs, in agreement with
446 previous reports (Matsunaga *et al.*, 2014).

447 The high level of synchrony of the eukaryotic communities among the 4 WWTPs was also
448 supported by the different correlation analyses described in Fig. 6 and Figs. S7-S9. Also, it is
449 important to note that the different interactions were not circumscribed to a specific kingdom,
450 as in each one of the 3 clusters, members of the 5 kingdom-like clades analysed were found. It
451 has been previously reported that a high level of relationships among eukaryotes is important
452 for the adequate maintenance of the ecological equilibrium of the food-web and the nutrient
453 cycling in a given ecosystem (Peng *et al.*, 2018). Consequently, the high level of co-occurrence
454 patterns observed in this work suggests that a highly imbricated structure of the eukaryotic
455 community is required for an effective WW treatment in this type of industrial WWTPs. These
456 results agree with those previously described by dos Santos *et al.* (2014), who analysed
457 protozoan and metazoan diversity in mixed WW (70% industrial and 30% domestic influents).

458 Also, it is remarkable the absence of human pathogens such as *Entamoeba* (Amoebozoa),
459 *Giardia* (*Fornicata*) or *Enterobius* (*Nematoda*) among the dominant genera in the AS. Similar
460 results were observed in intermediate treatment stages and effluent samples of different
461 domestic WWTPs by Zahedi *et al.* (2019), while other authors reported a high presence of these
462 pathogens in urban WWTPs effluents (Ma *et al.* 2016).

463 4.3 Roles of the core phylum-like taxa in WWTPs

464 Salinity increases are usually linked to a worsening in the system performance (Pham and
465 Nguyen, 2020; Cristóvão *et al.*, 2016). However, the driving mechanisms of this common
466 behaviour are generally unknown, and the effect of salinity over eukaryotic populations, which
467 play a key role in wastewater treatment, has been rarely studied. Due to the scarce number of
468 previous scientific works evaluating the relationships between the performance of WWTPs and

469 the eukaryotic diversity of their AS, potential roles of the core phylum-like taxa are proposed in
470 this work mostly according to their life-styles described in other ecosystems.

471 The shared phylum-like clades within the paraphyletic group of Protista, in which
472 relationships remain poorly understood in terms of diversity and systematics (Santoferrara *et*
473 *al.*, 2017), were Alveolata, Amoebozoa, *Ciliophora*, *Euglenozoa* and Other_Protists. In general,
474 protists are ubiquitous microorganisms (Galal *et al.*, 2018) of central importance in the
475 functioning of microbial food-webs, where they play diverse trophic roles (McManus and
476 Santoferrara, 2013) and subsequently, they are of vital importance in WWTPs. The members of
477 the supergroup Alveolata, which includes a large range of trophic modes and habitats, act as
478 important marine primary producers (Guillou *et al.*, 2008). Amoebozoa comprises organisms
479 with different feeding behaviours (bacterivory, detritivory, predatory and mixotrophic)
480 (Macumber *et al.*, 2020), and several previous works described its occurrence in WWTPs
481 (Chouari *et al.*, 2017; Cohen *et al.*, 2019). *Ciliophora*, also known as ciliates, are major
482 environmental micropredators with grazing activity that could directly stimulate bacterial
483 colonies (Johansson *et al.* 2004), and have been regarded as a main eukaryotic group in different
484 WWTPs (Peng *et al.*, 2018; Šimek *et al.*, 2019). *Euglenozoa* or flagellates have been described in
485 AS from WWTPs (dos Santos *et al.*, 2014; Yoshino *et al.*, 2017), where their occurrence could be
486 linked to their heterotrophic and phagotrophic life-style.

487 Fungal taxa shared in the samples analyzed this study were the phylum-like groups
488 *Ascomycota*, *Blastocladiomycota*, *Microsporidia*, *Unclassified_Fungi*, and other minority groups.
489 Nowadays, it is well established that fungal communities are prime movers in WWTPs as they
490 are potentially important contributors to various functions (Niu *et al.*, 2017; Wang *et al.*, 2017).
491 In addition to their beneficial roles for sewage treatment, fungi can be also involved in bulking
492 and foaming phenomena, resulting in poor settling and dewatering of AS in some cases (Hossain,
493 2004). *Ascomycota* display a high diversity of life-styles (Aranda, 2016) and they can contribute
494 to organic compounds' biodegradation, the aggregation of sludge flocs and detoxification (Niu

495 *et al.*, 2017); accordingly, their biodiversity and abundance are of great importance in sewage
496 treatment. *Ascomycota* was recognized as the most abundant fungal phyla in urban WWTPs in
497 several previous studies (Maza-Márquez *et al.*, 2016; Niu *et al.*, 2017; Assres *et al.*, 2019);
498 however, in this work, the prevalence of this phylum was minoritarian. *Blastocladiomycota*
499 includes saprotrophs or parasites of plants, animals or other fungi, and they usually account for
500 a minor fraction of the fungal community in WWTP bioreactors (Maza-Márquez *et al.*, 2016;
501 Zhang *et al.*, 2019), as also observed here.

502 Remarkably, a large quantity of fungal sequences remained unclassified at the phylum-like
503 level and were part of the dark matter fungi (Fig. 4 and Table S2), whose biodiversity and ecology
504 are largely unknown (Ryberg and Nilsson, 2018). A high correlation between NaCl concentration
505 and the RAs of dark matter fungi was revealed, which has not been previously reported in
506 industrial WWTPs. In this sense, the F4 facility, which bore the highest salinity, was particularly
507 enriched in unclassified fungi (37% of the overall eukaryotic community). Extreme environments
508 harbour a wider content of uncharacterized microorganisms whose occurrence indicates many
509 undiscovered microbial niches and interactions (Nobu *et al.*, 2015). Therefore, there is still a gap
510 in the knowledge of the fungal diversity and population interactions in fish-canning WWTP, and
511 the relevance of the links among fungal population shifts and salinity needs to be further
512 explored.

513 Inside the *Metazoa* kingdom, *Arthropoda*, *Cnidaria*, *Nematoda*, *Platyhelminthes*, *Rotifera*
514 and other minority phyla (*Annelida*, *Chordata*, *Mollusca* and *Porifera*) were shared among the 4
515 WWTPs. The different phyla within the *Metazoa* could be responsible for the improvement of
516 WWTP performance by controlling the density of bacteria by predation, reducing sludge
517 production, and enhancing sludge flocculation (Wang *et al.*, 2017). *Arthropoda* are members of
518 the eukaryotic community of full-scale WWTPs (Ju *et al.*, 2014; Bedoya *et al.* 2019), where they
519 play a major role in the food-webs in two ways, by decomposing organic material for themselves,
520 and by providing substrates for other eukaryotes via cracking through internal digestion

521 (Dehghani *et al.*, 2016). The majority of *Cnidaria* members have predatory life-styles, but certain
522 species may also scavenge dead animals (Frazão *et al.*, 2012). Their presence and roles in WWTP
523 bioreactors are not fully established but could be linked to the performance of a control over the
524 microbial populations. *Nematoda* and *Platyhelminthes* include microbotrophics (ingesting
525 bacteria, algae, single-celled fungi and protozoa) or predaceous lifestyles on other eukaryotes
526 (Noreña, *et al.*, 2015). Several works have earlier described the presence of both phyla in AS
527 from different urban and industrial WWTPs (Leal *et al.*, 2013; El Fels *et al.*, 2019). The highly
528 abundant phylum *Rotifera* encompasses predatory or microphagous organisms (Wallace *et al.*,
529 2015). They are a promising tool for controlling the over-proliferation of filamentous bacteria in
530 AS diminishing sludge bulking (Pajdak-Stós *et al.* 2017), and they are often found in industrial
531 WWTPs (dos Santos *et al.*, 2014). The minority phyla *Annelida*, *Chordata*, *Mollusca* and *Porifera*,
532 are mainly related to organic carbon content reduction in sewage treatment (Zhao *et al.*, 2010),
533 and the occurrence and roles of most of them are not yet well-established in WWTPs.

534 The shared phyla from the *Viridiplantae* and related kingdom-like rank were *Chlorophyta*,
535 *Rhodophyta*, *Stramenopiles* and *Streptophyta*. *Chlorophyta* have a high potential for
536 improvement of WWTP performance, being able to reduce organic matter, remove N and P
537 nutrients and heavy metals (Ariesyady *et al.*, 2016; Schulze *et al.*, 2017; Peralta *et al.*, 2019), and
538 generate O₂ through photosynthesis (Chen *et al.*, 2017). It is not surprising that the abundance
539 of this phylum was highly correlated to salt concentrations (Table S3 and Fig. 7) as they naturally
540 occur in seawater, indicating that their members are halotolerants and can be pivotal players in
541 WWTPs subjected to high salinity sewage, as it is the case of fish-canning WWTPs. *Rhodophyta*
542 usually are employed as microhabitats for diverse invertebrates and other algae (Aguirre *et al.*,
543 2017) and their presence in WWTPs could theoretically increase the diversity of eukaryotes. The
544 existence of *Rhodophyta* in WWTPs is scarcely reported, as only the work of Ghosh and Love
545 (2011) previously found them abundant in WWTPs. This shows the highly specific biodiversity of
546 the community found in the analysed WWTPs, probably due to the particular conditions of fish-

547 canning influents. Stramenopiles can act as main grazers of bacteria and play key roles within
548 the microbial food-web. Although the presence of this group in WWTP bioreactors has been well
549 demonstrated previously (Chouari *et al.*, 2017; Cohen *et al.*, 2019), in this work, only unclassified
550 sequences were found and, subsequently, a description of their hypothetical roles cannot be
551 inferred.

552 Finally, 6 minority phylum-like clades (*Cercozoa*, *Choanoflagellata*, *Heterolobosea*,
553 *Jakobida*, Rhizaria and Unclassified_eukaryotes, Fig. 4 and Table S2) were grouped together in
554 the hodgepodge Other Eukaryotes kingdom-like group. The shared phylum-like clades included
555 *Cercozoa*, *Heterolobosea*, Rhizaria and Unclassified_eukaryotes. The presence of both Rhizaria
556 and *Cercozoa* were previously described at the different steps of the treatment process in
557 WWTPs (Peng *et al.*, 2018; Hirakata *et al.*, 2019), where they are able to reduce N and P
558 concentrations in WW (Zahedi *et al.*, 2019). Besides, the members of Rhizaria are mostly
559 predatory heterotrophs (Cavalier-Smith *et al.*, 2018). Most heteroloboseans are bacterivores;
560 also, they are able to feed on different eukaryotes such as diatoms, or even on themselves in
561 a cannibalistic manner. Although they have been related to hypersaline environments (Park
562 *et al.*, 2007), no robust correlations between their abundance and NaCl content were found in
563 this work (Table S3). Some of the Unclassified eukaryotes identified in the AS samples have
564 been recently proposed as predators (*Ancoracysta*, Janouškovec *et al.*, 2017) or grazers
565 (*Telonema*, Yabuki *et al.*, 2013, and *Katablephari*, Kwon *et al.*, 2017). The presence of these taxa
566 in WWTPs was only previously confirmed for the genus *Anurofeca* (He *et al.*, 2017).

567 4.4 Effect of NaCl on eukaryotic biodiversity

568 The PCA (Fig S1) pointed out a stronger influence of the NaCl concentration in both WW and
569 AS over other abiotic variables and, similarly, the role of salt stress on the shaping of the
570 eukaryotic community structure was demonstrated in the NMS analysis (Fig. 7). Therefore, NaCl
571 concentration was a major driver of the shifts in the structure of the eukaryotic community. In
572 this sense, the changes in salinity resulted in a modulation of the RAs of the different phylum-

573 like clades rather than a change in the phyla that formed the eukaryotic community (Table 3 and
574 4). Recently, Rodríguez-Sánchez *et al.* (2019) reported that community structures of *Eukarya*
575 were affected by the salinity level more than by other factors and were more responsive to its
576 changes than the prokaryotic communities in two hybrid moving-bed pilot scale bioreactors and
577 a membrane bioreactor treating urban WW. Similarly, other studies showed that salinity was a
578 driving factor for the shaping of the eukaryotic community in different aquatic ecosystems
579 (Rojas-Jiménez *et al.*, 2019). On the other hand, although several authors highlighted the
580 negative effect of NaCl on the abundance of different phylum-like clades (Horikoshi *et al.*, 2010;
581 Cortés-Lorenzo *et al.*, 2016; Rodríguez-Sánchez *et al.*, 2019), strikingly, no strong negative
582 correlations were observed in this work (Fig. 7, Table S4). Taking into account the origin of the
583 raw material treated in this type of WWTP, it is not surprising that the eukaryotic populations
584 were well adapted to cope with salinity. In this sense, different mechanisms to protect
585 eukaryotic cells of the negative effect of NaCl have been developed by eukaryotes (Fuentes *et al.*
586 *al.*, 2016; Harding and Simpson, 2018; Vashishtha and Meghwanshi, 2018). Besides, Pham and
587 Nguyen (2020) previously reported that the efficiency of CAS systems was significantly affected
588 under NaCl contents >3% due to cell lysis. The results presented here indicate that a broader
589 eukaryotic diversity could reduce the negative effect of NaCl and promote the colonization of
590 the different ecological niches.

591 4.5 Bioindicator genera in fish-canning WWTPs

592 Several authors have proposed different eukaryotic genera as bioindicators of an optimal
593 performance of WWTP bioreactors; i.e., members of the *Breviatea* (genus *Subulatomonas*), and
594 *Chlorophyta* (genus *Desmodesmus*) (Chouari *et al.*, 2017; He *et al.*, 2017; Hirakata *et al.*, 2019).
595 Similarly, the occurrence of the genus *Arcella* (Amoebozoa), *Geotrichum* (Ascomycota) and
596 *Tokophrya* (Ciliophora) have been associated with deterioration of WWTP effluent quality (dos
597 Santos *et al.*, 2014; Zhang *et al.*, 2016). However, none of the abovementioned genera
598 accounted for a significant fraction of the eukaryotic communities in the fish-canning treatment

599 samples analysed here. Therefore, it is necessary to propose eukaryotic genera that can be used
600 as bioindicators in industrial WWTPs that operate under high salinity conditions.

601 As earlier stated in section 2.7, *Oocystis* (phylum *Chlorophyta*) is a core genus-like clade in
602 the 8 amplicon libraries here analysed, and its RA was strongly correlated to increasing values
603 of NaCl concentrations of both WW (Table S4). *Oocystis* are microscopic coccoid green algae
604 often described in many aquatic ecosystems (Štenclová *et al.*, 2017), and Huang *et al.* (2012)
605 described members of this genus as primary populations that efficiently reduce the
606 concentration of dissolved N compounds in different saltwater systems. Another advantage of
607 the presence of *Oocystis* in WWTPs is their ability to tolerate high levels of heavy metals (Soldo
608 and Behra 2000). Therefore, *Oocystis* may contribute to the maintenance of the ecological
609 equilibrium of the food-web and the nutrient cycling in WW subjected to high concentrations of
610 NaCl or heavy metals. Therefore, the high prevalence and ubiquity of *Oocystis* across the 4 fish-
611 canning WWTPs and its enrichment under increasing NaCl concentrations suggests that this
612 genus is a good candidate to be proposed as bioindicator in WWTPs of fish-canning facilities.

613 This work was pioneer in the analysis of global eukaryotic diversity in fish-canning WWTP.
614 Taking into account all data, the results here described significantly contribute to improve the
615 understanding of the ecology and putative functions of the different members of the eukaryotic
616 communities in industrial WWTPs operated under varying levels of salinity, since studies related
617 to this topic are currently scarce. In general, the putative contributions of eukaryotes into the
618 food-web could be acting as decomposers, primary producers or as predators/grazers of
619 bacteria, small metazoans and other eukaryotes. Other works are necessary in order to confirm
620 the specific contributions of different genera to the optimal operation of these industrial
621 WWTPs. Gathering diversity information from this and future work will help to elucidate the
622 ecology of the different members of the domain *Eukarya* and enable the proposal of more
623 bioindicator taxa useful to evaluate the operational state of industrial WWTPs. Despite the
624 advances in the knowledge of the ecology and metabolic roles of eukaryotes in sewage

625 treatments in those anthropogenic ecosystems, further research is required in order to better
626 understand the interactions among the abiotic and biotic components in WWTPs that will help
627 to establish new policy recommendations that contribute to the adequate management of saline
628 influents in fish-canning or other industrial WWTPs.

629

630 **5. Conclusions**

631 The eukaryotic communities in the AS of 4 fish-canning WWTPs differing in NaCl
632 concentration were successfully characterized by using an Illumina-based massive parallel
633 sequencing approach, which provided a high level of resolution and revealed a diversity wider
634 than previously recognized for this Domain in other WW treatment systems. This suggests that
635 the high-strength and salinity of the influents could require a higher number of eukaryotic
636 groups than in other WWTPs. The structures of the eukaryotic communities were highly
637 imbricated, since a common core of 25 phylum-like clades was shared among the four fish-
638 canning WWTPs, although the particular WW characteristics and the operating parameters in
639 each facility produced a narrow selection of the dominant eukaryotic OTUs. Amid the different
640 abiotic variables analysed, NaCl concentration was the major factor driving the shaping of the
641 eukaryotic communities, modulating the RAs of the different phylum-like clades of the common
642 core. The genus *Oocystis* was proposed as a bioindicator of the global eukaryotic community in
643 saline WWTPs, since it was the main core population whose RA was most intimately correlated
644 to NaCl concentration. Hence, monitoring the abundance of *Oocystis* spp. in industrial WWTPs
645 aids to evaluate the response of the eukaryotic communities to salinity. The results of the
646 present work contribute novel insights on the ecology of the eukaryotic communities in WWTPs
647 subjected to salinity, revealing their putative roles as decomposers, primary producers, and
648 predators/grazers of bacteria and other eukaryotes. These results provide a strong basis for
649 future studies delving into the analysis of their contribution to organic matter removal in these
650 industrial facilities. Finally, our study could be useful for fish-canning WWTPs increasing the

651 knowledge of the response of the systems at different salt concentrations that could help to
652 establish in future studies a predictive model between microbiome and operating conditions.

653

654 **Credit authorship contribution statement**

655 **David Correa-Galeote:** Formal analysis, Investigation, Methodology, Software Data
656 curation, Writing - original draft, Visualization. **Alba Roibas:** Formal analysis, Investigation,
657 Writing - original draft, Visualization. **Anuska Mosquera-Corral:** Funding acquisition, Term,
658 Conceptualization, Resources, Writing - review & editing, Visualization. **Belen Juárez-Jiménez:**
659 Term, Conceptualization, Resources, Writing - review & editing, Visualization. **Jesús González-**
660 **López:** Funding acquisition, Term, Conceptualization, Resources, Writing - review & editing,
661 Visualization. **Belen Rodelas:** Investigation, Term, Conceptualization, Resources, Formal
662 analysis, Writing - review & editing, Visualization, Supervision.

663

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668

669 **Declaration of competing interest**

670 The authors declare no conflicts of interest competing financial interests or personal
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672

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941 **Figure Captions**

942 Figure 1. Flowchart of the processes carried out in the four sampled WWTP for the treatment of fish-
943 canning effluents. a) F1, low salinity and conventional activated sludge (CAS) system, b) F2, moderate
944 salinity and CAS system, c) F3, moderate salinity and anaerobic reactor for biogas production and CAS
945 system for nitrogen removal, d) F4, high salinity and anaerobic reactor for biogas production and CAS
946 system for nitrogen removal. Thin arrows represent the water treatment line, thick arrows the sludge line
947 and dashed arrows gas lines.

948 Figure 2. Average relative abundances of operational taxonomic units (OTUs) assigned to domain level
949 from activate sludge samples retrieved from WWTPs treating fish-canning wastewater by Illumina high-
950 throughput sequencing (n = 2). Sequences reads that were not classified into any domain were labelled
951 as “Unclassified”. According to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$), different letters
952 indicates significant differences among samples for a given domain.

953 Figure 3. Average relative abundances of operational taxonomic units (OTUs) assigned to kingdom-like
954 level of the Eukaryotic domain in activate sludge samples (n=2) by Illumina high-throughput sequencing
955 of WWTPs treating fish-canning wastewater.

956 Figure 4. Relative abundances (represented by the areas of dots) of different phylum-like clades in
957 activated sludge samples (n=2) of WWTPs treating fish-canning wastewater identified by high-
958 throughput Illumina sequencing. Eukaryotic groups representing at least 0.5% of the total sequences or
959 at least a 0.25% for a given sample were shown. Other_Protist includes the sequences related to
960 *Diplonemea* class, *Petalomonadida* order, *Liburna* and *Carpediemonas* genera and unclassified protists;
961 Other_Fungi includes *Basidiomycota*, *Chytridiomycota* and *Mucoromycota* phyla; Other_Metazoa
962 includes *Annelida*, *Chordata*, *Mollusca* and *Porifera* phyla. Other_Eukaryotics include the sequences
963 belonging to *Cercozoa*, *Rhodophyta* and *Bacillariophyta* phyla, *Aphelida* and *Rhizaria* groups, and
964 Unclassified Eukaryotes.

965 Figure 5. Heatmap of the average clustering of the relative abundance of the phylum-like clades
966 identified in the DNA isolated from activated sludge samples (n=2) from WWTP bioreactors treating fish-
967 canning industrial wastewater identified by high-throughput Illumina sequencing.

968 Figure 6. Hierarchical Spearman correlation matrix diagram of the 37 different groups found in the
969 activated sludges samples (n=2) in WWTPs treating fish-canning wastewater identified by Illumina high-
970 throughput sequencing.

971 Figure 7. Nonmetric Multidimensional Scaling (NMS) analysis of the relative abundance eukaryotic
972 phylum-like taxa (relativized to the maximal taxon abundance) in activated sludges sample (n=2) retrieved
973 from fish-canning industrial WWTPs by Illumina sequencing and its linking to the main physicochemical
974 properties ($\log(X + 1)$ transformed) of influents and activated sludges of the WWT plants (volatile
975 suspended solids of activated sludge, VSS_AS; pH of wastewater, pH_WW; pH of activated sludge, pH_AS;
976 NaCl concentration in wastewaters, NaCl_WW; NaCl concentration in activated sludges, NaCl_AS; volatile
977 fatty acids in wastewater, VFA_WW; ammonium concentration in wastewater, NH_4^+ _WW). Samples from
978 F1: triangles; F2: diamonds; F3: circles; F4 samples: squares.

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