1   **A multivariate approach of changes in filamentous, nitrifying and protist**

2   **communities and nitrogen removal efficiencies during ozone dosage in a full-scale**

3   **wastewater treatment plant.**

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13   **Abstract:**

14   The application of low ozone dosage to minimize the problems caused by filamentous

15   foaming was evaluated in two bioreactors of an urban wastewater treatment plant.

16   Filamentous and nitrifying bacteria, as well as protist and metazoa, were monitored

17   throughout a one-year period by FISH and conventional microscopy to examine the

18   effects of ozone application on these specific groups of microorganisms. Multivariate

19   data analysis was used to determine if the ozone dosage was a key factor determining the

20   low carbon and nitrogen removal efficiencies observed throughout the study period, as

21   well as to evaluate its impact on the biological communities monitored. The results of

22   this study suggested that ozonation did not significantly affect the COD removal

23   efficiency, although it had a moderate effect on ammonia removal efficiency.

24   Filamentous bacteria were the community most influenced by ozone (24.9% of the

25   variance explained by ozone loading rate), whilst protist and metazoa were less affected

26   (11.9% of the variance explained). Conversely, ozone loading rate was not a factor in

27   determining the nitrifying bacterial community abundance and composition, although this

28   environmental variable was correlated with ammonia removal efficiency. The results of

29   this study suggest that different filamentous morphotypes were selectively affected by

30   ozone.

> The results of our study suggest that the structure of microbial communities significantly changed with ozone addition and different filamentous morphotypes are selectively affected.

34   **1. Introduction**

35   Bulking and foaming problems are frequent causes of effluent quality loss in

36   wastewater treatment plants (WWTPs) with activated sludge (AS) systems. These

37   episodes, caused by the proliferation of filamentous bacteria in the aerated tank, affect

38   the sludge settling properties (Jenkins et al., 2004), producing the occurrence of residual

39   solids in the secondary effluent. When problems became recurrent or chronic the

40   excessive growth of filamentous bacteria can be controlled by non-specific methods such

41   as continuous low ozone addition (Van Leeuwen and Pretorious, 1988). However, it has

42   been suggested that the effectiveness of oxidative treatments is regarded to cell membrane

43   composition and its permeability, particularly in bulking systems where hydrophobic

44   filaments are abundant (Seka et al., 2001).

45   Ozonation is a particularly interesting technology for wastewater treatment. The

46   strong oxidation potential of ozone contributes to solubilisation of suspended solids (SS)

47   and extracellular polymeric substances (EPS), cell lysis, oxidation and mineralization of

48   organic matter when it is applied to the AS process (Chu et al., 2009). Ozone also reacts

49   with inert solids, oxidizing and transforming them into biodegradable forms. During the

50   sequential decomposition processes produced by the ozonation in AS, organic matter and

51   debris are released from the microbial cells (Komanapalli and Lau, 1996). The leakage

52   of these soluble substrates results in an increase of soluble chemical oxygen demand

53   (sCOD), nitrogen and phosphorous loading rates in the reactor (Demir and Filibeli, 2012;

54   Sui et al., 2011), producing indirect impacts on microorganisms during ozonation

55   (Yapsakli et al., 2010). These potential impacts are in addition to direct effect of

56   ozonation on microorganism activity and mortality rates, which depend on each species

57   and their location in the floc (Bölhler and Siegrist, 2004; Fall et al., 2017). Previous

58   research (Fall et al., 2017; Isazadeh et al., 2014; Yan et al., 2009a) highlights the potential

59   of ozonation to transform the microbial community, thus impacting the overall WWTP

60   performance.

61   Among these issues, there has been a major concern about the effect that ozone

62   treatment can cause in nitrification processes. Nitrification is the oxidation of ammonia

63   and nitrite to nitrate by chemolithotrophic bacteria, mainly ammonia-oxidizing bacteria

64   (AOB) and nitrite-oxidizing bacteria (NOB). These bacterial groups are well known for

65   their slow growth rate and high sensitivity to inhibitory compounds and environmental

66   factors. Negative effects of ozonation on nitrification have been reported by several

67   authors (Gardoni et al., 2011; Naso et al., 2008; Richardson et al., 2009; Romero et al.,

68   2015), but only a few studies provide information about the impact of ozonation on the

69 structure of nitrifying bacterial communities (Chen et al., 2017; Izahadeh et al., 2014;

70 Levén et al., 2016; Yan et al., 2009a). In most of these studies, despite negative effects,

71 the nitrogen elimination ratios reached were enough to ensure good effluent quality,

72 indicating that ozonation and biological nitrogen removal are still compatible

73 technologies (Sui et al., 2014).

74   To date, most of the studies related with ozone treatment, have focused on the

75 optimization of operational conditions and have been conducted on pilot plants over short

76 experimental periods. This was because ozonation in full-scale WWTPs is both a

77 challenging operational process and energy intensive (Semblante et al., 2017). These

78 studies have provided a better understanding of the effects that ozonation treatment has

79 on microbial communities and the reactions that take place. Nevertheless, longer ozone

80 applications will likely have a greater influence on AS processes (Dytczak et al., 2008),

81 as the performance of WWTP is the result of a complex interaction between

82 environmental factors, influent conditions and operational variables. Therefore, it is

83 necessary to elucidate which combination of factors contributes to the stability of the

84 nitrification process when combined with ozonation.

85 In this study we propose a new approach to evaluate the impact of long-term ozone dosage

86 on microbial community composition (filamentous bacteria, nitrifying bacteria, protist

87 and metazoa), as well as on biological process efficiency in a full-scale AS system. In

88 order to assess the contribution of environmental variables to the variability observed in

89 microbial community structure and nitrifying performance, distance-based linear models

90 (DISTLM) were used.

91   **2. Materials and methods**

92 2.1.   WWTP and ozone system description

93   AS samples were collected from aerated reactors of a full-scale urban WWTP in

94   Spain. The WWTP consists of two independent wastewater treatment lines (CT1, CT2)

95   with A/O configuration, which together treat up to $34157 m^3$ wastewater per day. Each

96   reactor contains a floating ozonation unit installed with the aim to control filamentous

97   foaming, caused mainly by *Gordonia* sp. The sludge ozonation system consisted of an

98   ozone generator (Turboxal, Air Liquide S.A., France) using pure oxygen as the feed gas.

99   This generator distributes ozone through an ejector downward into the aerated basin,

100   producing a gas/liquid emulsion in the mixed liquor. Ozone dosage was regulated in the

101   aerated basins by controlling the oxygen input flow. Both treatment lines where

102   monitored throughout one-year period. The ozone was applied continuously during 180

103   days in CT1. In CT2 an ozonation period of 90 days was followed by non-ozonised period

104   of 45 days, after that ozone was applied 45 more days. During the study period the system

105   was operated with different ozone concentrations, varying dosages from 0 to $47.8 \, kgO_3/d$,

106   which corresponds from 0 to $0.15 \, gO_3/kgMLSS \cdot h$.

107   2.2.   Physico-chemical parameters and operational variables

108   Samples from aerated tank, influent and treated effluent were collected every fifteen

109   days during a year. The average characteristics of biological process operational

110   variables, analysed physico-chemical parameters, and treatment efficiencies are shown in

111   the supplementary material. Samples for determining soluble components were

112   immediately filtered after sampling using 0.45 µm glass-fibre filters. All the physico-

113   chemical parameters were examined in accordance with Standard Methods for the

114   Examination of Water and Wastewater (APHA, 2005). BOD samples were determined

115   by respirometry using Oxitop® system. Sludge volumetric index (SVI) was determined

116   according to Jenkins et al. (2004).

117   2.3.   Biological variables

118   For fluorescence *in situ* hybridization (FISH) analysis samples of mixed liquor

119   collected every fifteen days throughout a one-year, were fixed within 24 h of sampling in

120   4% paraformaldehyde for 3 h at 4°C, washed with PBS and stored in PBS/Ethanol (50%)

121   at −20 °C until analysis.

122   FISH was used to identify and quantify the nitrifying bacterial community and

123   filamentous bacteria present in AS from both reactors monitored in this study.

124   Hybridization was performed at 46°C according to Daims et al. (2004) with hybridization

125   times of 2-4 h for all oligonucleotide probes used in this study (Supplementary data S2).

126   Hybridized samples were examined with an Olympus BX50 microscope (Olympus, Paris)

127   equipped with 100W mercury high-pressure bulb and set filters U-MWB, U-MWIB and

128   U-MWIG.

129   In order to determine the relative abundance of AOB and NOB thirty images,

130   randomly selected, were captured per sample with camera Olympus DP70 for probe-

131   conferred fluorescence. MATLAB routine developed by Borrás, (2008) was used for

132   image processing and data analysis.

133   The identification and estimation of filamentous bacteria abundance was performed

134   by conventional microscopy before FISH, within 48 h of AS sampling. The identification

135   of morphological and structural characteristics where conducted according to Eikelboom

136   (2000, 2006) and Jenkins et al. (2004). Filamentous bacteria were quantified according

137   to a subjective scoring of filament abundance, ranging from 0 (none) to 5 (abundant)

138   (Eikelboom, 2000).

139   Sludge samples for determining protist and metazoan abundance were analysed

140   within 24 hours sampling. Two replicates of 25 μl were analysed to survey the density of

141   protist and metazoa, by direct counting, using phase contrast microscopy. Four additional

142   replicates were performed for the following organisms: *Epistylis* sp., *Opercularia* sp. and

143   *Carchesium* sp. Different staining procedures, dry silver nitrate (Klein, 1926), silver

144   carbonate impregnations (Fernández-Galiano, 1976) and Flutax-2 staining (Arregui et al.,

145   2003), were carried out to confirm several ciliate species. Two groups of naked amoebae

146   were considered based on their cell size (> 50 μm and < 50 μm) (Zornoza, 2017).

147   Organisms were identified by using identification guides (Foissner et al., 1991, 1992,

148   1994, 1995).

149   2.4.   Multivariate analysis

150   In order to elucidate which possible factors correlate with nitrifying populations,

151   filamentous bacteria, protist and metazoan dynamics (biological variables), and

152   biological process performance (explained variables) multivariate data analyses was

153   used. Explanatory matrix was constructed with 51 environmental variables (physico-

154   chemical variables of influent, effluent and mixed liquor) and 6 operational parameters

155   of the process. Distance-based linear modelling (DISTLM) procedure was used; this

156   multivariate multiple regression routine uses a resemblance matrix of biological process

157   performance multivariate results and species abundance data to regress against to the

158   explanatory matrix (Anderson et al., 2008). Statistical analyses were performed for both

159   reactors together (CT1 and CT2) as they have the same process and similar operational

160   conditions but different ozone dosage.

161   To avoid background noise, those biological variables with occurrence higher than

162   20% (protists and metazoa) were selected for the multivariate analysis (Pérez-Uz et al.,

163   2010). Prior to analysis, count data were transformed. Square root (nitrifying and

164   filamentous bacteria) and logarithmic (protists and metazoa) transformation were used to

165   down-weight the effect of high occurrence of certain species, whilst still enabling

166   sufficient signal from rarer taxa to be observed (Clark et al., 2014). Environmental

167   variables were log-transformed and normalized prior to multivariate data analyses

168   (euclidean similarity). A Bray-Curtis resemblance matrix was created from the

169   transformed data (Bray and Curtis, 1957). Draftsman plots and correlation matrix were

170   produced to assess the distribution of each variable and to identify multicollinearty. In

171   the pairs of variables with a Pearson's correlation coefficient of 0.85 or larger, one of the

172   variables was excluded from the analysis (Clarke et al., 2014).

173   DISTLM was constructed by using stepwise forward selection procedure. Akaike's

174   information criterion corrected (AIC_C) and Bayesian information criterion (BIC) were

175   selected in order to obtain the most parsimonious model, identifying the simplest

176   reproductions with the greatest explanatory power (Anderson et al., 2008). We use

177   distance-based redundancy (dbRDA) routine to visualize a constrained ordination on the

178   tailored values from the best model (Anderson et al., 2008). Analysed variables were

179   represented as bubble where variable value is represented by bubble size. The strength

180   and direction of the relationship between the explanatory variables and biological

181   variables (Pearson values >0.45) were presented as vectors to visualize the ordination.

182   Six statistical models were employed to evaluate the correlation between

183   environmental variables and the considered explained variables (carbon removal

184   performance, nitrogen removal performance, filamentous bacteria, nitrifying bacteria and

185   protist and metazoan communities). Three of them were constructed to reach a better

186   understanding of possible correlations influencing nitrogen removal efficiencies and

187   nitrifying bacteria population dynamics. The first was used to investigate which

188   explanatory variables influence the observed changes in nitrogen removal efficiencies;

189 the second was used to find the correlations between the nitrifying bacteria populations

190 and the environmental variables; the third examines the relations between the relative

191 abundance of nitrifying bacteria and nitrogen removal efficiency.

192   All multivariate analyses were performed with the statistical software package

193 PRIMER 7 (Clarke and Gorley, 2015) and PERMANOVA+ (Anderson et al., 2008).

194   **3. Results and discussion**

195 3.1.   Carbon removal performance

196   The effect of ozonation on effluent quality has been an issue of major concern since

197 ozone affects microbial activity and viability, solubilizes the SS and enhances the

198 biodegradability of inert solids, which results in increased sCOD (Chu et al., 2009). With

199 the aim of observe the biological process removal efficiency separately from the

200 settlement process efficiency, values of sCOD removal efficiency (sCODre) were

201 determined. sCODre ranged from 45% to 90% and 38 to 90% for CT1 and CT2,

202 respectively, and remained above 80% in both reactors during ozonated period.

203 Examination of the relationships between environmental variables and organic matter

204 removal efficiencies did not show any significant correlation between ozone dosage and

205 carbon removal performance (Table 1). The results of our study coincide with those

206 reported by other studies (Lee et al., 2005; Dytczak et al., 2007; Nie et al., 2014), which

207 found that ozonation did not significantly affect the COD removal efficiency (CODre),

208 despite of the wide ranges of ozone concentrations tested. DISTLM showed that 19% of

209 the variation in CODre could be attributed to dissolved oxygen concentration (DO) in the

210 reactor and sludge retention time (SRT). Therefore ozonation did not appreciably affects

211 the COD removal capacity. This might be mainly attributed to the high biodegradability

212 of the organic material released during SS solubilisation that is promptly used as substrate

213 by heterotrophic bacteria (Dytczak et al., 2007; Nagare et al., 2008).

214 3.2.   Nitrogen removal performance and nitrifying bacterial community

215   The effluent total nitrogen (TN) and NH_4^+ -N concentrations were similar in

216 treatment lines, ranging from 20 to 54 mg/L (mean value = 35 mg/L) and from 4.4 to 45

217 mg NH_4^+ -N /L (mean value= 27 mg NH_4^+ -N /L) respectively. These values were the

218 result of low nitrogen removal efficiencies (TNre) observed through the studied period.

219 The TNre and NH_4^+-N removal efficiency (NH_4^+-Nre) varied from 16 to 57% (mean value

220 = 40%) and 10 to 90% (mean value = 44%) respectively for CT1, whereas these values

221 for CT2 ranging from 20 to 65% (mean value = 44%) and 15 to 63% respectively (mean

222 value = 41%).

223   AOB and NOB were detected during all the studied period despite of the low NH_4^+

224 -Nre achieved. The average relative abundance of these species was 3% for AOB and 1%

225 for NOB (Fig. 2). *Nitrosomonas oligotropha* lineage and members of the genus *Nitrotoga*

226 were found by FISH as the dominant nitrifiers responsible for ammonia and nitrite

227 oxidation, respectively. Halophilic and halotolerant *Nitrosomonas* spp., as *N. eutropha*

228 *and N. halophila,* and members of the genus *Nitrospira* were present at lower relative

229 abundance. All of them are common nitrifying species founded in AS nitrogen removal

230 systems. FISH probe signals were not detected for *N. europea, Nitrosococcus mobilis,*

231 *Nitrobacter, Nitrolancetus hollandicus*, Subcluster Thaumarchaeota group I.1b and

232 *Candidatus Nitrosopumilus maritimus*. In Fig. 2, where the relative abundance of

233 nitrifying populations together with O_3LR is illustrated, non-obvious patterns are found

234 between the two parameters. The model (Table 1) used to investigate which explanatory

235 variables influence the observed changes in nitrogen removal efficiencies showed that

236   41% of the variation in nitrogen removal performances was explained by using four

237   environmental variables. Ozone loading rate (O_3LR) was the best explanatory variable

238   that predicted its behaviour (18.6 % of variance explained), followed by MLSS, PO_4^+ -P

239   and organic loading rate (OLR) expressed as sCOD. The model results were visualized

240   using a dbRDA (Fig. 1a). In order to detect the variables showing best correlations to the

241   set of environmental variables, nitrogen removal efficiencies and nitrogen species on

242   effluent were superimposed as vectors. Total of 32.5% variation was explained by the

243   axis 1, being soluble total nitrogen (sTN) and NH_4^+ -N the variables with highest

244   correlation coefficients (r = 0.83 and r = 0.73 respectively). These results indicate that

245   sTN and NH_4^+-N in the effluent were positively correlated with the increasing O_3LR (Fig.

246   1a). NH_4^+ -Nre and dissolved Kjeldahl nitrogen removal efficiency (sTKNre) also present

247   relevant correlations with axis 1 (r = -0.60 and r = -0.56, respectively) indicating that best

248   removal efficiencies were correlated with O_3LR lower values. The results of the

249   sequential test (Table 1) provide information about the environmental variables (MLSS,

250   PO_4^+ -P and OLR) that can contribute to balance the negative effects of ozone in nitrifying

251   removal performance. DISTLM used to evaluate the correlations between nitrifying

252   bacteria populations and environmental variables, showed that ozone is not the variable

253   that contributes to the highest percentage variance explained (4.8%) (Table 1). Oils and

254   fats loading rate (OFLR) (6.9%) and chemical oxygen demand in mixed liquor (CODML)

255   (6.8%) where the variables mostly correlated with the environmental variables. OFLR

256   were correlated with the axis 1, explaining 14.4% of the variation, however genera

257   *Nitrosomonas* was the only taxa that correlates significantly with the axis 1 (r=-0.55). The

258   model shows that O_3LR was explaining the 4.8% of the variation of nitrifying bacterial

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259 communities, but no relevant correlation was found by dbRDA with AOB or NOB

260 species.

261   Multivariate data analysis for environmental interpretation of relations between

262 nitrogen removal performances and nitrifying bacteria shows that 22% of nitrifying

263 bacterial communities variation can be related to sTN removal efficiency (sTNre) (13.1%

264 explained variance) and the percentage of NO_2-N in effluent (8.8% explained variance).

265 Percentage of NO_2-N was introduced as a variable in order to detect if there were any

266 factors determining the presence of NO_2 on effluent. It was noticed by the dbRDA (data

267 not shown), that the sTNre correlates with the relative abundance of *N. oligotropha* and

268 *Nitrotoga*, corresponding to dominant nitrifiers in the studied period.

269   The results obtained in our study using multivariate analysis indicate that ozone had

270 a limited impact on NH_4^+ -Nre, and no strong correlations were found between ozone

271 dosage and the nitrifying bacterial community dynamics. Therefore, the impact of ozone

272 on the nitrifying bacterial structure does not explain at all the low ammonia removal

273 efficiency observed, although relationship between dominant species and removal

274 efficiencies were found. Levén et al. (2016) evaluated the effect of low ozone dosage to

275 control *M. parvicella* on nitrifying bacterial community structure in a full-scale plant. In

276 this study, no relevant variations on nitrifying bacterial community composition or

277 abundance were found for the different ozone dosages tested (4.4 – 6.6 gO_3/kgSS).

278 Conversely, in the study by Levén et al. (2016) nitrogen removal performances were also

279 unaffected by the ozone application, probably because other factors balancing the

280 potential effects of ozone. Discrepancies between different studies are possibly due to

281 variations in sludge properties and experimental conditions (Semblante et al., 2017; Yan

282   et al., 2009a), due to the fact that the ozone effect is dependent on the ozonisation

283   conditions (Gardoni et al., 2001; Richardson et al., 2009; Meng et al., 2013).

284   It is well known that nitrifiers form compact aggregates as stress protection (de

285   Boer et al., 1991). *Nitrospira* has been found as dominant NOB in other ozonised AS

286   systems (Isazadeh et al., 2014; Levén et al., 2016). Previous reports noticed that this NOB

287   present higher resistance to ampicillin than *Nitrobacter and* other heterotrophs (Spieck et

288   al., 2006), probably due to its tendency to aggregate in dense flocs embedded in an EPS

289   matrix, that offers them protection against the inhibitors. Yan et al. (2009b) observed that

290   at high ozone dosage (80 mg O_3/g SS) some Gram negative bacteria, typically found in

291   WWTP structured in tetrads or clusters, resisted to the oxidative attack. These findings

292   imply that the presence of an EPS matrix might protect the nitrifying bacteria against the

293   oxidative effect. Other authors (Böhler and Siegrist 2004) suggested that nitrifiers are

294   normally overgrown by faster-growing heterotrophs and may therefore be partly

295   protected in the sludge floc and not exposed to ozone as much as the heterotrophs.

296   3.3.   Filamentous bacteria

297   Conventional microscopy and FISH analysis were conducted in order to identify

298   and quantify 11 filamentous bacteria morphotypes, present in AS samples of both reactors

299   (Fig. 3). FISH probes used for the study are presented in the supplementary material.

300   *Gordonia sp. Haliscomenobacter hydrosis*, Type 0092, Type 0803-I (industrial

301   phylotype), Type 0803-D (domestic phylotype) and Type 1851 were the most dominant

302   filamentous bacteria observed during the studied period. As showed in Table 1, the degree

303   of variation explained for filamentous bacteria by the set of environmental variables was

304   60.6%. Highest correlations with environmental variables and the set of explanatory

305   variables were found for O_3LR and temperature in the reactor (Tr), indicating that ozone

306 plays an important role structuring the filamentous bacterial community. Results from the

307 multivariate data analysis revealed significant differences in the response of filamentous

308 bacteria to ozone and Tr. Type 0803-I, Type 0041, Type 1851 (r>0.45) declined in

309 abundance as ozone increased and temperatures decreased. The opposite pattern between

310 variables was associated with the occurrence of *Gordonia sp*. and T0803-D. Type 0803-

311 I was the most strongly associated with ozone (r =-0.71), indicating that this type was

312 most affected by ozonation, in contrast to *Gordonia* sp. which seemed to be less affected

313 when ozone was applied at concentrations up to 47.8 kgO_3/d, as reflected in the dbRDA

314 (Fig. 1b), which corresponds with a dosage of 0.15 $gO_3/kgSSVLM \cdot h$. *Gordonia* sp. as

315 well as 0803-I type are both bacteria that grow into the floc matrix, but their occurrence

316 has an opposite trend, indicating there are other factors determining ozone resistance. In

317 this study, the oxidizing agent did not further affect the filaments that grow outside flocs

318 more than other filaments present in the floc matrix, as observed in other studies

319 (Caravelli et al., 2006).

320   In previous studies lower ozone doses were effectively used to control filamentous

321 bulking (1.4 to 2.8 gO_3/kg SS) (Lyko et al., 2012; Nilsson et al., 2014; Ried et al., 2014;

322 Saayman et al., 1996), than ozone dosage applied in this study. Different factors as

323 process configuration, floc characteristics and ozone dosage point, probably influenced

324 the observed results. The study of Guo et al. (2012), where the influence on chlorination

325 or cetyltrimethyl ammonium bromide (CTAB) was evaluated on 021N type, suggests that

326 the most important factor influencing the ozone treatment success seems to be the cell

327 wall penetration capacity of the components. Previous studies have demonstrated that

328 different morphotypes are selectively affected by the chemical treatment applied (Nilsson

329 et al., 2018; Seka et al., 2001). In our study, the observed resistance of *Gordonia* sp. to

330 ozone treatment can be the result of mycolic acids presented in the cell wall of these

331 organisms, since these acids are strongly hydrophobic. Goi et al. (1998) considered that

332 the *Gordonia* foaming suppression mechanism of ozone, within a range of ozone below

333 3 mg/l, was due to the decomposition of mycolic acid and not because the number of

334 *Gordonia* was reduced by oxidative action of ozone. These findings are similar to those

335 reported where *M. parvicella* and *Nostocoida limicola* presented resistance to chemical

336 treatments due to their hydrophobic cell walls (Seka et al., 2001). Therefore it would be

337 of particular relevance to determine the penetration capacity before to implementation of

338 non-specific filamentous control treatment.

339 3.4.   Protist and metazoan dynamics.

340   It is well known that protist and metazoa play an important role in maintaining a

341 good balance in AS treatment systems. These populations consume excess free bacteria

342 decreasing turbidity and stimulating their growth, as well as they functioning as a

343 bioindicators in the AS process. Twenty-six species of protist and three species of

344 metazoa were identified. The most common protists quantified were small flagellates and

345 amoebae (Fig. 4).

346   According to the DISTLM results (Table 1), the most important structural factors

347 for the protist and metazoan community dynamics were O_3LR (11.9% of variance

348 explained), OFLR (2.2 % of variance explained) and SRT (2.9% of variance explained).

349 dbRDA on Fig. 1c shows that almost all the protist and metazoan species presented a

350 negative correlation with ozone concentration, indicating that ozonation significantly

351 impacted on this community. Only *Tokophyra infusionum* and *Aspidisca cicada* presented

352 positive correlations, although they were not statistically significant. Protists less tolerant

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353   to ozone addition were *Acineria uninata* (r = -0.62), A*moebae* (r = -0.50), *Opercularia*

354   *articulata* (r = -0.48) and *Vorticella microstoma* (r = -0.48).

355   Only few studies (Nilsson et al., 2018; van Leeuwen et al., 2009; Yan et al., 2009a),

356   have evaluated the effects of ozone addition on protist and metazoan communities. Van

357   Leeuwen et al. (2009) did not find evidence of harmful effects of ozone on protist

358   communities, meanwhile Yan et al. (2009a) found an increase of 1.57 in the number of

359   protist and metazoa in a lab-scale ozonised reactor in contrast with the non-ozonated

360   reactor. They attributed this increase to the direct composition of cell debris from cellular

361   lysis, which probably leads to propagation of microfauna. Nevertheless, in this study it

362   has been concluded that full-scale studies are necessary to evaluate the degree of ozone

363   impact in these populations, since the differences found between reactors may be the

364   consequence of other factors or changes in the influent characteristics. The results of our

365   study are in agreement with those from Nilsson et al. (2018), as they observed certain

366   sensitivity of these communities when ozone concentrations were above 3.0 to 4.8

367    $gO_3/kgTSS$.

368   **4. Conclusions**

369   Ozone treatment did not significantly affect the structure of nitrifying communities,

370   although it was linked to its functional attributes, being ozone the most significant

371   environmental correlated variable with NH_4^+-Nre.

372   This study suggested that different filamentous morphotypes are selectively

373   affected by ozone, and highlights the importance of determining the factors influencing

374   the ozone treatment resistance. From an implementation perspective, the concentration

375   applied should be investigated prior to full-scale application

376   Multivariate data analysis is an interesting tool to evaluate, as particular reference

377   for an specific process, which environmental variables can contribute to balance the

378   negative effects of ozone in nitrifying removal performance.

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380   E-supplementary data of this work can be found in online version of the paper.

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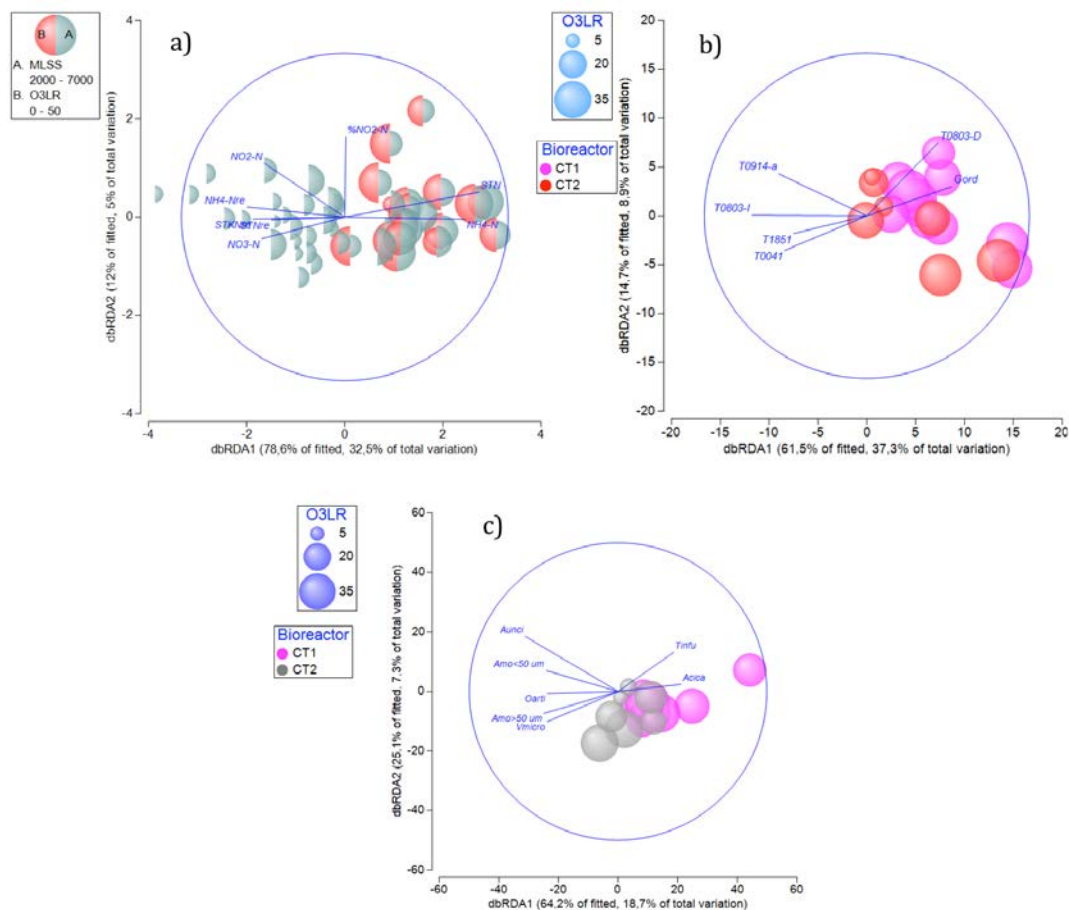
546

547   Fig. 1. dbRDA plots representing: (a) model of temporal variation on nitrogen

548   species on effluent and nitrogen removal efficiencies in relationship to environmental

549   variables, (b) model of temporal variation on filamentous bacterial community in

550   relationship to environmental variables and (c) model of temporal variation on protist

551   and metazoan community in relationship to environmental variables. Bubbles are scaled
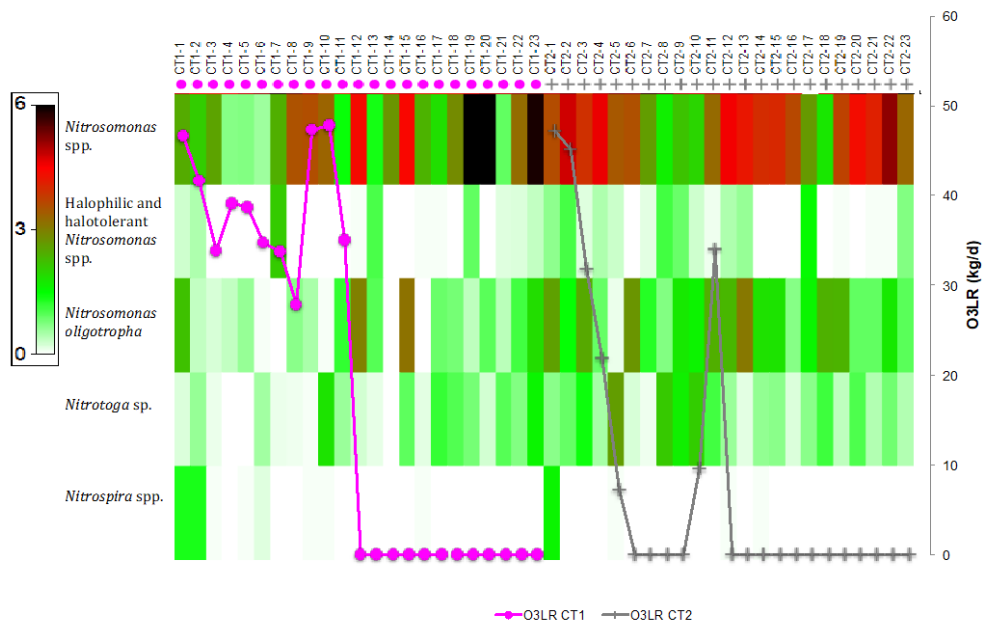
552   to represent the value of the explanatory variables. Vectors show the direction and

553   strength of the correlation between species and explanatory variables. MLSS, mixed

554   liquor suspended solids (mg/L); O3LR, ozone loading rate (kgO3/d).
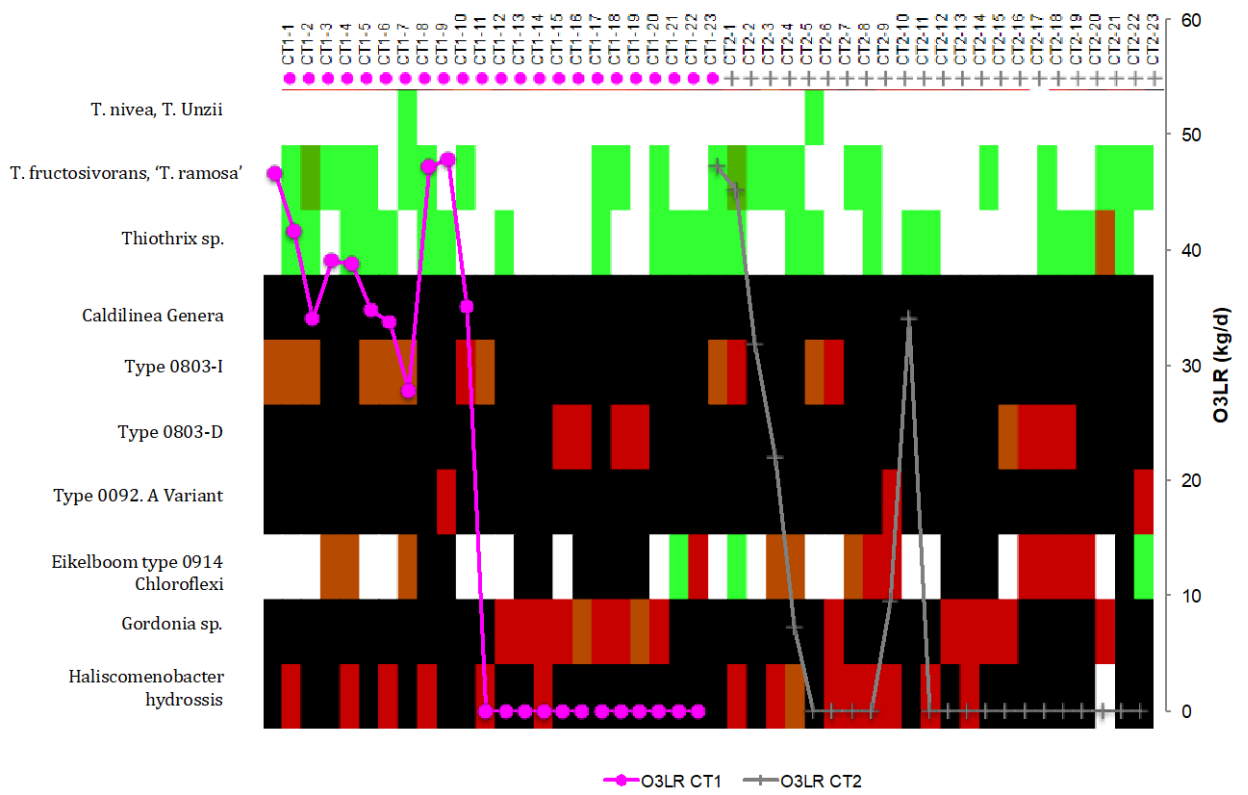
555

556   Fig. 2. Relative abundance of nitrifying bacterial community and ozone loading rate

557   (O3LR) in the reactors 1 (CT1) and 2 (CT2).
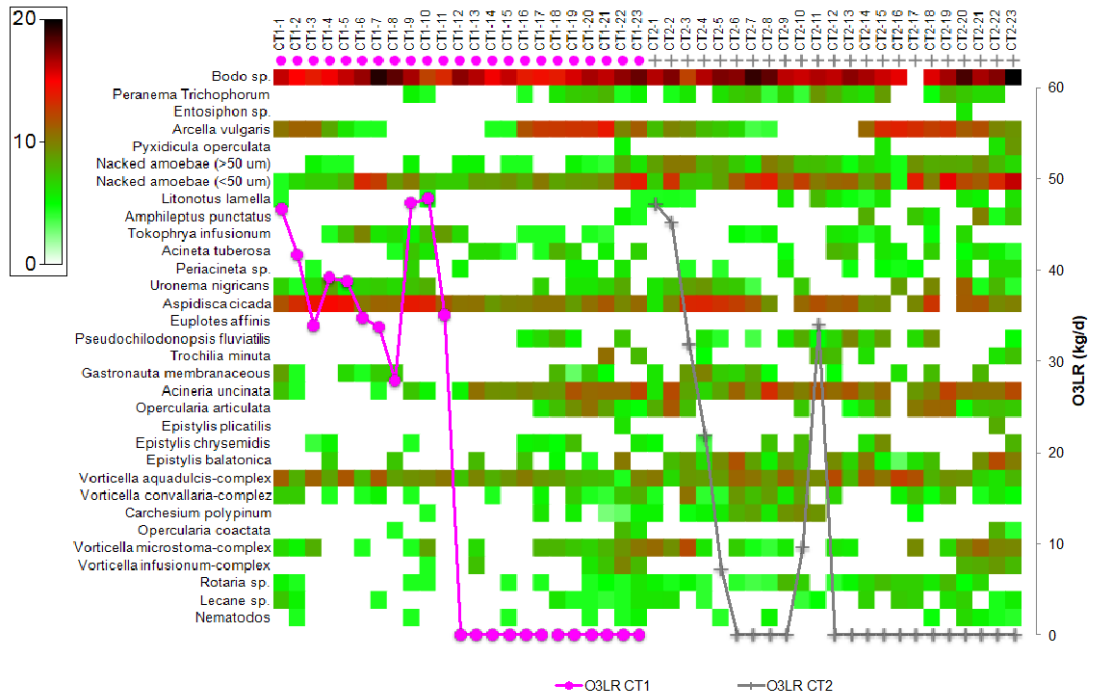
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559

560   Fig. 3. Filamentous bacteria abundance and ozone loading rate (O3LR) in the

561   reactors 1 (CT1) and 2 (CT2).

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563

564   Fig. 4. Protist and metazoan abundance and ozone loading rate (O3LR) in the

565   reactors 1 (CT1) and 2 (CT2).

Table 1.
Results of multivariate multiple regression analyses (DistLM)

1) Operational variables and carbon removal performance				
Environmental variables		Sequentially		
	AICc	Pseudo-F	p	% Prop.
HO	99.692	4.6785	**0.021**	9.611
SRT	96.499	5.4459	**0.015**	10.161

2) Environmental variables and nitrogen removal performance				
Environmental variables		Sequentially		
	BIC	Pseudo-F	p	% Prop.
O$_3$LR	92.809	10.084	**0.001**	18.645
MLSS	92.446	4.1027	**0.012**	7.0861
PO4-PLR	90.502	5.6149	**0.004**	8.7581
OLR (COD)	89.304	4.7344	**0.004**	6.7817

3) Environmental variables and relative abundance of nitrifying bacteria				
Environmental variables		Sequentially		
	AICc	Pseudo-F	p	% Prop.
OFLR	283.74	3.2839	**0.016**	6.9452
MLCOD	282.54	3.3867	**0.011**	6.7939
O$_3$LR	282.3	2.4818	**0.05**	4.8127
PO4-PLR	281.95	2.6492	**0.027**	4.9433

4) Nitrifying bacteria and nitrogen removal performance				
Environmental variables		Sequentially		
	AICc	Pseudo-F	p	% Prop.
STNre	280.56	6.6631	**0.001**	13.152
%NO2-N	277.91	4.8746	**0.001**	8.8429

5) Environmental variables and protist and metazoa				
Environmental variables		Sequentially		
	BIC	Pseudo-F	p	% Prop.
O$_3$LR	307.19	5.9873	**0.0001**	11.978
OFLR	305.74	5.2366	**0.0001**	2.1533
SRT	304.88	4.5025	**0.0001**	2.9131

6) Environmental variables and filamentous bacteria				
Environmental variables		Sequentially		
	BIC	Pseudo-F	p	% Prop.
O$_3$LR	1271.8	14.565	**0.001**	24.869
NTLM	406.71	5.0908	**0.002**	7.9532
T^{a}r	334.49	4.5305	**0.002**	6.5408
F/M (COD)	349.55	5.2089	**0.001**	6.8353
MLSS	270.26	4.3572	**0.003**	5.2849
CPT	286.16	5.0846	**0.001**	5.5957
CarbLR	180.66	3.4082	**0.013**	3.5327

Percentage proportion of variance in species or biological removal efficiencies data explained by that variable. Significant values (<0.05) are indicated in bold.

566