1   **Title:** Assessment of toxic effects of magnetic particles used for lake restoration on

2   *Chlorella* sp. and on *Brachionus calyciflorus*

3   **Authors:** I. Álvarez-Manzaneda[1,2] and I. de Vicente[1,2*]

4   **Affiliations:** (1) Departamento de Ecología, Facultad de Ciencias, Universidad de

5   Granada, 18071 Spain

6   (2) Instituto del Agua, Universidad de Granada, 18071 Spain

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11   **Full address for correspondence**:

12   [*]Corresponding author.

13   Departamento de Ecología, Facultad de Ciencias, Universidad de Granada, 18071 Spain

14   Phone: (+34) 249768; Fax: (+34) 958 243093 ; email: ivicente@ugr.es

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Abtract

Laboratory tests, by following standardized Organization for Economic Co-operation and Development (OECD) protocols, were run for evaluating the acute effects of iron magnetic microparticles (MPs), recently proposed for lake restoration, on *Chlorella* sp. (algal growth) and on the rotifer *B. calciflorus* (mortality). In addition, the MPs potential indirect effects on rotifer egg bank were assessed by performing hatching rate test with *B. calciflorus* cysts in contact with dissolved iron (Tot-Fe$_{dis}$). In the algal growth test, no inhibition occurred at the two lowest MPs concentrations (0.01 and 0.05 g l^{-1}) which would correspond, considering the adsorption efficiency ratio (Phosphorus: MPs), to P concentrations lower than 0.94 mg P l^{-1}, much higher than typical concentrations found in natural waters. For higher MPs dose (EC$_{50}$ for *Chlorella* sp. was 0.15 g l^{-1}), no nutrient limitations but high turbidity and Tot-Fe$_{dis}$ values cause negative effects on algal growth. For the case of *B. calciflorus*, LC$_{50}$ was 1.63 g MPs l^{-1} (corresponding to 30.7 mg P l^{-1}). When analyzing Tot-Fe$_{dis}$ effect, the hatching rate of *B. calciflorus* cysts was 100% for all treatments. To sum up our results for *B. calciflorus* acute and chronic toxicity tests, it is extremely unlikely the mortality of adult organisms in contact with MPs as well as an affectation of the rotifer egg bank. In conclusion, it is expected that MPs addition in a real whole-lake application cause minor lethal and sublethal effects on both *Chlorella* sp. and *B. calciflorus*.

Keywords: magnetic particles, *Chlorella* sp., *Brachionus calciflorus*, toxicity, eutrophication, lake restoration

1. Introduction

48 Phosphorus (P) translocation from its land reserves to the aquatic environment is a

49 direct consequence of the impact of human action on the environment which lastly

50 drastically affect to the biogeochemical P cycle (Cordell et al., 2011). Hence, we are

51 facing two coupled and worldwide increasing problems: (i) the global food resources

52 depletion link to the reduction of P reserves essential for making fertilizers P (Gilbert,

53 2009) and (ii) the eutrophication, nutrient enrichment, of aquatic ecosystems (OECD,

54 1982; Sas, 1989; Cooke et al., 2005).

55 Eutrophication is the leading cause of water pollution for many freshwater and coastal

56 marine ecosystems and is a rapidly growing problem in the developing world (Harper,

57 1992; Schindler, 2006; Smith and Schindler, 2009). Because of the main limiting

58 nutrient for the aquatic primary production is P, it is essential to reduce P concentration

59 when restoring eutrophicated systems. It is well accepted that P availability in lake

60 water can be reduced by three ways (Hupfer and Hilt, 2009): (i) Reducing P external

61 load, (ii) Increasing P retention by the sediment and (iii) Increasing P export from the

62 system. Among these techniques, the control of P external load is an essential and

63 preliminary step before implementing any other management strategy (Smith, 2009;

64 Jeppesen et al. 2009). In fact, controlling catchment-derived nutrient loading is a

65 prerequisite for lasting lake restoration efforts, otherwise internal stocks of nutrients

66 will be replenished (Cooke et al., 2005).

67 As a result of the two above mentioned problems (the depletion of P reserves and the

68 eutrophication of aquatic ecosystems), new and innovative methods are required. In this

69 context, iron (Fe) magnetic particles (MPs) have been recently proposed as convenient

70 P adsorbent (de Vicente et al., 2010; de Vicente et al., 2011; Merino-Martos et al.,

71 2011). Briefly, MPs are used to adsorb P from aqueous solutions and after the

72 adsorption is carried out, P loaded MPs can be separated by using a high gradient

magnetic separation process. Later, P can be desorbed and potentially used as a fertilizer while the bare MPs can be reused. Next, we summarize the most relevant advantages for using MPs as P adsorbent for lake restoration (de Vicente et al. 2010; Merino-Martos et al. 2011; Funes et al. 2016, 2017; Álvarez- Manzaneda et al. 2017): (i) high P adsorption capacity under both batch and flow conditions; (ii) the insignificant dependence on physico-chemical conditions (redox and pH) of their P adsorption; (iii) the reduction in sedimentary P_{Mobile} concentrations caused by their addition (under both oxic and anoxic conditions), potentially contributing to a long-term reduction in P efflux; (iv) their lesser cost in comparison to other P adsorbents (e.g. $AlCl_3 \cdot 6H_2O$ and Phoslock®); and (v) the low toxic effects on plankton and benthic organisms. Accordingly, the use of MPs would help to counteract both the depletion of P reserves and the eutrophication of aquatic systems by removing P from eutrophicated systems and by using the recovered P as a fertilizer.

However, it is clear that before adding MPs in a whole-lake application strategy it is essential to gain more knowledge about MPs potential toxic effects on lake biota. The procedures currently in use for conventional risk assessment have a first step that consists in the identification and characterization of hazards based, among others, in basic toxicity tests (Amiard-Triquet et al., 2015). Accordingly, acute and chronic effects of MPs on *Daphnia magna* and on *Chironomus* sp. have been already evaluated (Álvarez- Manzaneda et al. 2017). However, and considering that MPs addition makes sense just in eutrophicated systems where the zooplankton community is dominated by rotifers instead of cladocerans (Gannon and Stemberger, 1978), it is essential to test MPs effects on rotifers. Apart from rotifers, algae were also chosen as test organisms in this study due to the following consideration: (a) they belong to the first level of the trophic chain and so, any change in the composition and density of the phytoplankton

could change the biological and chemical quality of an ecosystem (Lewis, 1995); (b) they seem to be more sensitive for some contaminants than animal species (Hoffman et al., 2003) and (c) they have a short life cycle, allowing the evaluation of toxic effects over several generations (Silva et al., 2009). In addition to basic toxicity tests, experimental designs mimicking a natural environment (microcosms) are also recommended (Caquet, 2013). Therefore, by using microcosms from an hypertrophic coastal lake, potential changes on species composition and abundance of phytoplankton (del Arco et al., unpublished) and on zooplankton community (Álvarez-Manzaneda et al., unpublished) after MPs addition have been assessed.

Although the majority of standardized ecotoxicity tests and biomonitoring in aquatic systems are based on the active component of invertebrate communities, dormant egg banks are crucial for the long term survival and community dynamics of many aquatic organisms (Navis et al., 2013). In fact, the invertebrate dormant egg banks in the sediments of aquatic ecosystems constitute ecological and evolutionary reservoirs of species (De Stasio, 1989; Hairston & Munns, 1984; Hairston, 1996). Among invertebrate communities, rotifers are important components of such egg banks in freshwater systems. Most planktonic rotifers reproduce via cyclical parthenogenesis (Snell & Janssen, 1995), incorporating both asexual (amictic) and sexual (mimic) reproduction into their life cycle (Preston and Snell, 2001). The application of MPs for lake restoration may involve two kind of interaction with lake biota: i) direct and short-term effect caused by MPs and ii) indirect and long-term effect caused by the dissolved Fe (Tot-Fe$_{dis}$; after MPs removal). Therefore, and for the case of rotifers, it is essential to assess the potential effect of MPs and Tot-Fe$_{dis}$ on both adult organisms and cysts.

In this context, our working hypothesis is that MPs addition for lake restoration cause lethal and sublethal effects on algae and on rotifers. Accordingly, in this paper we

123 combine both acute, which are mostly based on mortality as endpoint, and sublethal

124 toxicity tests looking at growth and/or reproduction of the biota. In particular, the

125 general aim of this paper was to assess, by laboratory tests and following standardized

126 Organization for Economic Co-operation and Development (OECD) protocols, the

127 acute effects of MPs on *Chlorella* sp. (algal growth) and on the rotifer *Brachionus*

128 *calyciflorus* (mortality). In addition, hatching test with *B. calyciflorus* cysts were

129 performed for assessment the MPs indirect effects due to the Tot-Fe$_{dis}$ increase. As MPs

130 are efficient P adsorbent and they may therefore affect nutrient availability for

131 phytoplankton, during the *Chlorella* sp. experiments a through monitoring of physico-

132 chemical changes in the aqueous solutions was accomplished.

133 **2. Material and methods**

134 *2.1. Test organisms*

135 Laboratory experiments were carried out with two species belonging to two different

136 trophic levels, the freshwater green algae *Chlorella* sp. and the rotifer *B. calyciflorus*

137 *Chlorella* sp. (cell volume: 365 μm^3; diameter: 8.8 μm) was selected as the test species

138 because this unicellular green alga has a good sensibility to toxicants and it is easily

139 cultured at laboratory (Silva et al., 2009).

140 The stock culture of *Chlorella* sp., provided by the Department of Ecology of the

141 University of Jaén, was cultivated in an 800 ml volume with Bold's Basal Medium

142 (BBM; Bold, 1949). This freshwater algae medium was chosen based on previous

143 studies who found that it is better than natural medium for toxicity tests with *Chlorella*

144 sp. (Polonini et al., 2015). The culture was maintained in an isolated room at a

145 temperature of 22 ± 0.5 ºC and a cycle of light: darkness of 16: 8 h. In order to avoid the

146 sedimentation of algae cells, the culture was shaken at 100 rpm and the cell density was

147 estimated by using a Neubauer counting chamber.

148   Rotifers are organisms frequently used in toxicity tests such as: i) acute toxicity tests

149   with mortality endpoints; ii) life-cycle tests and short-term toxicity tests and iii) tests on

150   suborganismic level (Dahms et al., 2011). Because of their sensitivity to toxic

151   compounds they are good toxicity indicators (Alvarado-Flores et al., 2012). In addition,

152   they often play a key role in the ecosystems dynamics, being useful as models in

153   ecotoxicology (Snell and Janssen, 1995). Rotifers are characterized by a short-life cycle

154   and a rapid reproduction (Fernández-Casalderrey et al., 1991) and their cysts can be

155   store dried for long time. All of these reasons make them very suitable for toxicology.

156   *B. calyciflorus* were used for toxicological tests by following the American Society for

157   Testing and Materials International (ASTM) standardized test (Allen, 1998). Cysts were

158   purchased dried (from MicroBioTests, Gent, Belgium) and they were kept, at darkness,

159   at a temperature of 5 ± 2ºC. Hatching was carried out about 16 h before the beginning of

160   the test, at 25ºC and under continuous illumination of 3000-4000 lux, in rotifer medium

161   (96 mg $NaHCO_3$, 60 mg $CaSO_4 \cdot 2H_2O$, 60 mg $MgSO_4 \cdot 7H_2O$ and 4 mg KCl per liter of

162   distilled water; Allen, 1998). Then, *Chlorella* sp., originated from a culture collection of

163   the University of Jaén, served as food for the stock rotifers with a density of $3x10^6$ cells

164   ml^{-1}, required concentration for assuring a high population growth (Sarma et al., 2001;

165   Lucía-Pavón et al., 2001) and a temperature between 20 and 25ºC. In addition, algal cell

166   concentration was estimated using Neubauer counting chamber, replacing and counting

167   the solution used as food for rotifers every week.

168   *2.2. General characterization of magnetic microparticles*

169   The composition of the micronsized Fe particles used is 97.5% Fe, 0.9% C, 0.5% O and

170   0.9% N, according to the manufacturer (BASF, Germany). Its magnetization properties,

171   electrophoretic mobility, particle size distribution and P adsorption properties have been

172   previously characterized (de Vicente et al., 2010; de Vicente et al., 2011; Merino-

173   Martos et al., 2011). Briefly, MPs have spherical shape, are relatively polydisperse and

174   with a mean diameter of 805±10 nm. They present a ferromagnetic behavior with a

175   negligible remnant magnetization as well as a thin oxide surface layer which determines

176   that surface charges are controlled by the pH in the aqueous medium (isoelectric point is

177   around pH 6.5).

178   *2.3. Toxicological test with* Chorella *sp*

179   *2.3.1. Growth inhibition test*

180   Growth inhibition test was made according to an OECD's modified protocol (1984) and

181   by using a sonicated (5 min) 50 g MPs l^{-1} stock solution.

182   First, each glass flask was inoculated with 25000 cells ml^{-1} from *Chlorella* sp. stock.

183   Next, different volumes of the MPs stock solution were added to each treatment for

184   getting a final concentration of: 0.01; 0.05; 0.1; 0.5; 0.7; 1; 1.5 and 2 g MPs l^{-1} in a final

185   volume of 100 ml. All treatments and controls (no MPs addition) were run in four

186   replicates. Test flasks were randomly placed on a horizontal shaker at 125 rpm for

187   avoiding   the   algae   precipitation.

188   After 24 h of contact time, MPs were removed by applying a magnetic field gradient

189   exerted by a permanent magnet (volume ¼ 25.6 cm^{3}; NB032, Aiman GZ, Spain).

190   Removal process of MPs was carried out by immersing the magnet twice for 3 s in the

191   vessels.

192   The experiment, which lasted for three days, was performed following the OECD

193   standardized protocol (1984) with a temperature between 24 and 25.5ºC and under

194   continuous illumination of 6660 lux. Algal cells concentration was measured five times:

195   before MPs addition; right after MPs removal and 24, 48 and 72 h after MPs removal. A

196   Nageotte's counting chamber was used due to the low cell density (Lund et al., 1958;

197   Sabiri et al., 2011; Gubelit et al., 2015).

198 Following the OECD protocol, the mean value of the cell concentration was plotted

199 against time to obtain growth curves. Next, the area below the growth curves was

200 calculated according to this formula:

201 $$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1}) \qquad (1)$$

202 where:

203 A: area

204 N_0: number of cells ml^{-1} at time t_0

205 N_1: number of cells ml^{-1} at t_1

206 N_n: number of cells ml^{-1} at t_n.

207 t_1: time of first measurement after beginning of the test.

208 t_n: time of the nth measurement after beginning of the test.

209 Finally, the percentage inhibition of the cell growth (I_A) was calculated as the difference

210 between the area under the control growth curve (A_c) and the area under the growth

211 curve at each concentration (A_t) according to the following equation:

212 $$I_A = \frac{A_C - A_T}{A_C} x100 \qquad (2)$$

213 For the estimation of the EC$_{50}$, I_A values were plotted on semilogarithmic paper against

214 the corresponding concentrations. The intercept of the regression line with the parallel

215 drawn to the abscissa at I_A was the EC$_{50}$ (OECD, 1984).

216 *2.3.2. Physical-chemical variables*

217 At the end of the experiment, physical-chemical variables were monitored.

218 Conductivity, pH and dissolved oxygen concentrations (DO) were measured by using a

219 multiparameter probe (Hanna Instrument, HI 9829) while turbidity was measured with a

220 turbidimeter ISO 7027 (LW-TN3024).

221 Ammonium (NH_4^+), nitrites (NO_2^-), nitrates (NO_3^-), Dissolved Inorganic P (DIP) and

222 Tot-Fe$_{dis}$ were also measured at the end of the test after filtration (Whatman GF/F). The

223   analytical methods were as follows: NH_4^+ was determined following the phenate

224   method (Rodier, 1989); NO_2^- were analyzed following the sulfanilamide method

225   (Rodier, 1989); NO_3^- were quantified by using the ultraviolet spectrophotometric

226   screening method (APHA, 1995); DIP was analyzed by the molybdenum blue method

227   (Murphy and Riley, 1962) and Tot-Fe$_{dis}$ was measured by using spectroscopy emission

228   by plasma of inductive coupling (ICP-OES PERKIN-ELMER OPTIMA 8300).

229   2.4. *Toxicological test with* Brachionus calyciflorus

230   2.4.1. *Mortality test with magnetic particles*

231   This toxicological test, which lasted for 24 h and with no feeding during the test, was

232   carried out following the ASTM's standardized protocol (Allen, 1998). First, from a

233   stock solution containing 5 g of MPs l^{-1}, we prepared the next test concentrations: 0.1;

234   0.5; 0.7; 1; 1.5; 2; 2.5; 3; 3.5; 4 and 5 g l^{-1} of MPs, which were sonicated for 5 min. All

235   treatments and controls (no MPs addition) were run with four replicates.

236   When placed in the recommended medium (Allen, 1998), rotifer cysts hatch in about 16

237   h at 25°C. Later, ten individuals (< 2 h) of *B. calyciflorus* were placed in each well of a

238   multiple-well plate (48 well plates. Total volume per well: 1.4 ml) by using a

239   stereomicroscope and a micropipette and, immediately, 1 ml of the different solutions

240   was added. The plate, covered with parafilm to avoid evaporation, was placed in a

241   culture chamber at darkness and at temperature of 25 ± 1ºC.

242   After 24 h of contact time with the MPs, the organisms were observed by using a

243   stereomicroscope and the number of living and dead organisms was recorded. If the

244   animal did not move the mastax or foot for 5 seconds it was recorded as a dead

245   individual. Rotifers mortality in control was always lower than 10%.

246   For estimating the LC$_{50}$ (concentration of MPs which cause the 50% of mortality of the

247   total organisms) in the *B. calyciflorus* test, Probit analysis was carried out with the

248   statistical program SPSS. This is a parametric statistical method very used in toxicology

249   to analyze doses-response tests transforming sigmoidal dose-response curves to a

250   straight line that can be analyzed (Vincent, 2014).

251

252   2.4.2.   *Cysts hatching in the presence of dissolved iron*

253   With this experiment, we aim on assessing the effect of the Tot-Fe$_{dis}$ (dissolved from

254   MPs)   on   the   cysts   hatching   in   *B.   calyciflorus.*

255   Firstly, suspensions containing different MPs concentrations (0; 0.1; 0.5; 0.7; 1; 1.5; 2;

256   2.5; 3; 3.5; 4 and 5 g l^{-1}) were prepared from an stock solution of 5 g of MPs l^{-1}. They

257   were sonicated for 5 min and after 24 h, MPs were removed by applying a magnetic

258   field gradient exerted by a permanent magnet (the same as described in section 2.3.1).

259   For running the toxicological experiment, one cyst was placed in each well of the multiple-well

260   plate (96 well plates. Total volume per well: 0.37 ml) containing 0.35 ml of each MPs

261   concentration and the plate was covered with parafilm to avoid evaporation. The multiple-well

262   plate was placed in a culture chamber at 3000-4000 lux of continuous illumination and at

263   temperature of 25±1ºC. All treatments were run with eight replicates. After 16 h, cysts were

264   observed every half hour until the last cyst hatched and the number of hatched cysts were

265   recorded. Finally, time of the first hatching (TFH; Gutierrez et al., 2017) and synchronized time

266   (Ts; Ortega-Salas, 2013) were calculated. In particular, Ts was estimated as the difference

267   between T$_{90}$ (time when 90% of cyst hatched) and T$_0$ (time when the first neonate

268   hatched).

269   *2.4.3.   Physical-chemical variables*

270   Temperature, hardness and pH were measured at the beginning and at the end of the test

271   while DO was measured just at the beginning. Tot-Fe$_{dis}$ concentrations were also

272   measured at the end of the experiment by using the method already described in the

273   section 2.3.2.

274   *2.5. Statistical data analysis*

275   In the algal inhibition test, As both non transformed and logarithmically transformed

276   data did not fit a normal distribution, non parametrical tests (Kruskal-Wallis ANOVA

277   and U of Mann-Whitney; software SPSS) were carried out for identifying if there exists

278   significant differences between control and treatments.

279

280   **3. Results and discussion**

281   *3.1. Effect of magnetic particles on* Chlorella *sp.*

282   In the algal growth inhibition test, no inhibition occurred at the two lowest MPs

283   concentrations (0.01 and 0.05 g l^{-1}) while the highest MPs concentration (2 g l^{-1}) caused

284   an average inhibition of 83%. The EC_{50} was very low (0.15 g l^{-1}; Fig. 1). When

285   comparing these results with those found for the same MPs and *Selenastrum*

286   *capricornutum* (EC_{50} of 1.5 g l^{-1}; Álvarez-Manzaneda et al., unpublished) we noticed

287   great differences which could be determined by the different chemical composition of

288   the solution (BBM was used in the present study while ISO medium was used by

289   Álvarez-Manzaneda et al. (unpublished). Similarly, Millington et al. (1988) noted that

290   the toxic values from algae toxicity tests are affected by the medium composition.

291   Although EC_{50} for *Chlorella* sp. was very low, this concentration was much higher than

292   others found for metals or nanoparticles (NPs) of titanium, zinc, aluminum or silica for

293   *Chlorella* sp. (Mehta and Gaur, 1999; Ji et al., 2011; Iswarya et al., 2015). In similar

294   studies performed with others green algae and NPs, EC_{50} was also lower than that found

295   in the present study (Christensen and Nyholm, 1984; Griffitt et al., 2008; Becaro et al.,

296   2014; Bhuvaneshwari et al., 2015; Adam et al., 2015). However, it is important to bear

297   in mind the limitations for these comparisons as eventually, toxic effects will

298   dramatically depend on the type of particle and on the alga specie. In this sense, Menard

299   et al. (2011) highlighted the great heterogeneity in the results obtained by using the

300   same NPs but different alga species. Even more, it is well known the close inverse

301   relationship between specific surface area of the particles and its toxic effect on the

302   organisms (Fujiwara, 2008; Van Hoecke et al., 2008; Navarro et al., 2008; Ji et al.,

303   2011; Clément et al., 2013) and so, it is risky to compare toxic effects of microparticles

304   and NPs.

305   Similarly to the estimations made by Álvarez-Manzaneda et al. (2017), and considering

306   the 53 mg MPs: mg P mass ratio as the adsorption efficiency ratio (de Vicente et al.,

307   2010; Merino-Martos et al., 2011), the addition of 0.15 g MPs l^{-1} (EC_{50} for *Chorella* sp.)

308   would correspond to a scenario of 2.8 mg P l^{-1}, which is an extremely high value for

309   typical inland waters (hypereutrophic category correspond to annual mean TP

310   concentration > 100 µg l^{-1}; Nürnberg, 1996). This highlights that the use of MPs in

311   effective concentrations for P removal is likely to have no effect or minor effects in

312   phytoplankton community.

313   For comparing the toxicity of MPs with other P adsorbents (Phoslock, alum, Zeolites,

314   calcite) used for lake restoration, a wide literature review has been done. Similarly, to a

315   recent study performed with *D. magna* and *Chironomus* sp. (Álvarez-Manzaneda et al.,

316   2017), an evident scarcity of well standardized tests makes difficult to establish a

317   thorough comparison. In fact, no previous studies have been done with *Chlorella* sp.

318   and other P adsorbents. For *Scenedesmus obliquus*, van Oosterhout and Lürling (2013)

319   found that the threshold Phoslock® concentration for causing a depletion in algal

320   biovolume was 0.5 g l^{-1}. Considering the 100:1 Phoslock®: P dose ratio, recommended

321   by the manufacturer (Reitzel et al., 2013), 0.5 g Phoslock l^{-1} would match to 5 mg P l^{-1}

322   which is a rather atypical P concentration in natural waters.

323   Factors affecting the growth of microalgae may be included in two categories:

324   environmental factors (physical) and nutritional factors (chemical). Physical factors

325   include pH, temperature, light intensity and the aeration of the system; while nutritional

326   factors comprise the composition and amount of the chemical species in culture medium

327   (C, N, P, among others; Daliry et al., 2017). Accordingly, during this experiment a

328   thorough chemical monitoring of the solutions was achieved for identifying the main

329   factor driving algal growth inhibition. No significant changes were observed between

330   control and treatment for DO (Tables 1 and 3). pH significantly increased when

331   increasing MPs concentration, except for the lowest MPs concentration; while

332   Conductivity was only significantly higher in the treatments with some of the highest

333   MPs concentration (1 and 2 g MPs l^{-1}) compared to control.

334   As expected, turbidity was significantly higher in treatments than in control (Table 2).

335   In fact, turbidity increased with MPs following a logarithmic law (r= 0.88; $p<0.05$; Fig.

336   2a). Therefore, algal growth inhibition could be due to the MPs "shading effect".

337   Previous studies have also shown inhibitory effects of NPs which trapped the cells (Ji et

338   al., 2011; Gong et al., 2011; Huang et al., 2016; Cupi and Baun, 2016). This "shading

339   effect" may mask or limit the chemical toxicity because algal cells with slow growth,

340   due to low light intensity, are less sensitive to toxics than those with faster growth

341   (Hjorth et al., 2016). Van Oosterhout and Lürling (2013) also observed a notably

342   increased in turbidity (up to 211 NTU) when adding Phoslock®. This shading effect

343   caused that algae growth rather than be affected by toxicity is affected by physical

344   inhibition (Sørensen et al., 2016). Our results evidence that algal growth inhibition was

345   higher than 80% when adding 0.5 g MPs l^{-1} which lastly caused extreme turbidity

346   values (>500 NTU). However, in a real whole-lake application, these negative effects

347   are unlikely to occur as 0.5 g MPs l^{-1} would correspond to extremely high P

348 concentration (9.4 mg l^{-1}) and secondly, because MPs are characterized by a fast

349 sedimentation rate (Funes et al. 2017) and so, turbidity quickly decreased with time. In

350 fact, del Arco et al. (unpublished) found that after just one hour, turbidity decreased

351 around 40%. Unexpectedly, turbidity values obtained in the present study are extremely

352 higher than those found by del Arco et al. (unpublished) when using the same MPs in

353 the same concentration range. The only difference which could explain so huge

354 variation in NTU values is the solution (medium) where MPs were dissolved: BBM in

355 the present study and commercial mineral water in del Arco et al. (unpublished).

356 Therefore, it is expected that in a real whole-lake application turbidity values will be

357 much lower than those found in the present study.

358 Nutrient concentration in the experiment was checked along the experiment. In relation

359 to DIN fractions, NH_4^+ did not show significant differences between control and

360 treatments; while marginal significant differences were found for NO_3^- and NO_2^-

361 concentrations (Table 2). As it was expected, because an algal growth culture medium

362 very rich in inorganic nutrients (BBM) was used for this experiment, DIN concentration

363 was much higher than that consider by Reynolds (1992, 1999) as limiting for primary

364 producers (80 µg N l^{-1}). In the case of DIP only significant differences between control

365 and the two highest concentrations of MPs were found (Table 2). It is important to note

366 that MPs caused a significant reduction in P concentration (Fig. 2b) but still P-SRP

367 concentrations were extremely higher than P threshold concentration (3 µg l^{-1}) proposed

368 by Reynolds (1992, 1999) for identifying P as a limiting nutrient of the primary

369 production. Even more, when estimating DIN:SRP molar ratio, an extremely low value

370 (< 0.02) was found for all cases, reflecting that *Chlorella* sp. was N limited. Similarly,

371 Wu et al. (2014) found that *Chlorella vulgaris* can grow with lower N concentrations

372 than P.

373   At last, Tot-Fe$_{dis}$ concentration was significantly higher in 1.5 and 2 g MPs l^{-1}

374   treatments than in control (Table 2). Average Tot-Fe$_{dis}$ concentration was 1.11 and

375   12.43 mg l^{-1} in control and treatments, respectively. These values are much higher than

376   other reported by Funes et al. (2016; 2017) when adding MPs, under anoxic and oxic

377   conditions, to natural waters. Therefore, our results reflect that MPs are much easily

378   dissolved in BBM (algal growth medium) than in natural waters. However, Tot-Fe$_{dis}$

379   concentrations are in the range of those used by Estevez et al. (2001) for studying iron-

380   dependent oxidative stress in *Chlorella vulgaris*. These authors found that, algal growth

381   was estimulated when Fe availability (EDTA:Fe, 2:1) was lower than 100 μM (5.6 mg Fe l^{-1});

382   while Fe concentrations higher than 200 μM (11.2 mg Fe l^{-1}) led to a drastic decrease in the

383   growth of the cultures. Similarly, we have found inhibition growth in *Chlorella* sp. higher than

384   80% for Tot-Fe$_{dis}$ > 10 mg l^{-1} (Table 2).

385

386   *3.2. Ecotoxicological tests with* Brachionus calyciflorus

387   *3.2.1.   Mortality test with magnetic particles*

388   LC$_{50}$ in *B. calyciflorus* was 1.63 g MPs l^{-1} (Figure 3) which is in the range of EC$_{50}$

389   (immobilization; at 24 h) values reported for other planktonic (1.99 g MPs l^{-1} for *D.*

390   *magna,* Álvarez-Manzaneda et al., 2017) and benthic organisms (1.57 g MPs l^{-1} for

391   *Chironomus* sp.; Álvarez-Manzaneda et al., 2017). Although LC$_{50}$ and EC$_{50}$ are

392   obviously different endpoints, Jones et al. (1991) noted that sublethal toxicant

393   concentration may change organism behaviour (in this case their mobility) conducting

394   to long-term consequences on the survival rates. Therefore, and especially for

395   planktonic organisms, there exists an explicit relationship between both endpoints. In

396   this sense, experiments with *D. magna* in contact with MPs have recently shown a

397   physical immobilization of the organisms (Álvarez-Manzaneda et al., unpublished)

398   caused by the attachment of the particles in their bodies decreasing the movement

399 capacity of the organism as others authors have found for other type of particles

400 (Skjolding, 2016).

401 There exists a clear inconsistency between our results from acute standardized tests and

402 those obtained from outdoor microcosm experiments (Álvarez-Manzaneda et al.,

403 unpublished). In fact, our results have shown that the presence of 1.5 g MP l^{-1} caused

404 the death of 40% of the population of *B. calyciflorus*; while when the whole plankton

405 community (microcosm experiments) was exposed to 1.4 g MP l^{-1} no significant effect

406 was observed neither in the zooplankton biomass or diversity. More specifically, in the

407 microcosm experiments no significant changes in *B. calyciflorus* abundance were

408 observed after 1.4 g MP l^{-1} addition. Similarly, Pascoe et al. (2000) in a comparative

409 study about toxicological effects of pollutants (three reference chemicals) in laboratory

410 and fields experiments found that No Observed Effect Concentrations (NOEC) was

411 lower in laboratory than in field, reflecting a higher organisms sensibility under

412 laboratory conditions.

413 At this point it is important to consider that there exists a continuum of experimental

414 contexts used in aquatic toxicology which ranges from single-species toxicity tests to

415 natural ecosystems (Caquet, 2013). Standardized laboratory tests, which are used for

416 determining environmentally safe concentrations of pollutants (Sih et al., 2004), can

417 have high throughput, their results can easily be interpreted and compared among

418 laboratories, and they often correctly predict lethal or sublethal toxic effects on natural

419 communities (Chalcraft et al., 2005; Versteeg et al., 1999). However, this approach

420 usually does not consider additive or synergistic effects of multiple biotic and abiotic

421 stress factors (Sih et al., 2004; Mikó et al., 2015). In fact, microcosm studies reflect

422 more environmentally realistic exposure conditions, including natural abiotic conditions

423 (OECD, 2006). Accordingly, it is evident that *B. calyciflorus* is much less vulnerable to

424 MPs under more natural conditions (microcoms) than in single-species toxicity tests and

425 therefore, in a future context of a whole lake application of MPs and considering a real

426 MPs dose, it is expected that MPs toxic effects on *B. calyciflorus* are negligible.

427 Experiences with other P adsorbents such as Phoslock® are likely to reveal a major

428 toxicity than MPs. In particular, van Oosterhout and Lürling (2013) found that *B.*

429 *calyciflorus* population growth notably decreased when Phoslock® concentrations were

430 higher than 0.2 g l^{-1}, which would correspond, following the same calculations

431 explained above in section 3.1., to 2 mg P l^{-1} (LC_{50} for MPs is 1.63 g l^{-1} matching to

432 30.75 mg P l^{-1}).

433 Despite of the limitations for comparing the toxicity of pollutants which ultimately

434 depend on many factors (e. g. valence of the element, redox conditions), our results are

435 likely to indicate that MPs have a lower toxicity for *B. calyciflorus* than other

436 compounds reported in the literature which cause abnormal behaviour on this species a

437 few hours after the contact (Charoy and Janssen, 1999). Similarly, Zweerus et al. (2017)

438 found a decrease in the population growth of *B. calyciflorus* when they were exposed to

439 copper concentrations (50 to 100 μg l^{-1}) much lower than MPs concentrations used in

440 the present study.() The LC_{50} obtained in our study is much higher than that found for

441 *B. calyciflorus* in contact with other compounds such as copper (26 μg l^{-1}; De

442 Schamphelaere et al., 2006), endosulfan (5.15 mg l^{-1}; Fernández-Casalderry et al.,

443 1992), lindane (8.5 mg l^{-1}; Ferrando et al., 1993) or lead (125 μg l^{-1}; Grosell et al.,

444 2006). When comparing our results for MPs with other metallic NPs, we found out that

445 LC_{50} is higher for MPs than for NPs (Manusadžianas et al., 2012) as a result of the

446 previously described strong dependency of toxicity on particle size. In fact, NPs have a

447 much smaller size than MPs (≈800 nm; de Vicente et al., 2010) penetrating in the wall

448 gut and tissues (Rothhaupt, 1990; Vadstein et al., 1993; Snell & Hicks, 2009).

449   3.2.2. Finally, it is important to consider that during the acute test, organisms were

450   exposed to the best conditions, except that they were unfed during the 24 h

451   experiment (as it is established in the standardized protocol). In this sense,

452   some authors have suggested that in presence of food these results could

453   have changed (Luna-Andrade et al., 2002; Perez & Sarma, 2008), although

454   food privation begins to cause mortality about 32 h at 25ºC (Allen, 1998).

455   *Cysts hatching in the presence of dissolved iron*

456   After 22 h, the hatching of *B. calyciflorus* cysts was 100% for all treatments (Figure 4)

457   and so, Tot-Fe$_{dis}$ released after MPs addition did not affect rotifer hatching rate.

458   Similarly, Ts and TFH were not significantly different between control and treatments

459   (Table 3). In particular, Ts ranged from 4.2 (treatments) to 5.2 h (control). Therefore,

460   this situation corresponds to optimal conditions for the rotifers hatching (Snell and

461   Persoone, 1989; Ortega-Salas, 2013). Some authors have noticed that the suitable

462   conditions for cysts rotifers hatching are just a minimum of light and oxygen

463   concentrations (Gilbert, 2017); while others authors have found the need to stimulate

464   the cysts more than once until their hatching (Martínez- Ruíz & García-Roger,

465   2015)The high hatching rate is in accordance with the extremely low Tot-Fe$_{dis}$

466   concentrations, ranging from 0 to 0.061 mg l^{-1} (Figure 5). These values were much

467   lower than those recorded as negative for the survival of *B. calyciflorus* (Couillard et al.,

468   1989; Santos-Medrano and Rico-Martínez, 2013). Tot-Fe$_{dis}$ concentrations were much

469   lower than those found in the *Chlorella* sp. experiment (section 3.1) when the same

470   MPs but different medium were used, thus confirming the relevance of the medium

471   where MPs are dissolved. In relation to the physical-chemical parameters, no significant

472   changes were observed at beginning and at the end of the test (Table 3).

473 Contrarily to our results, previous studies have evidenced the sensibility of *Brachionus*

474 *patulus* and *B. calyciflorus* to metals such as cadmium, copper or chromium, which

475 drastically affect the production of offspring (Sarma et al., 2006; Gama-Flores et al.,

476 2007). Similarly, insecticides significantly affect the survival and reproduction of

477 rotifers (Ferrando et al., 1996).

478 All in all, although there exist a clear lack of studies focused on assessing the effects of

479 other substances discharged to the natural environment on cysts hatching of *B.*

480 *calyciflorus*, we may conclude that MPs effects are likely to be negligible. In fact, the

481 null effect on hatching rate of rotifer cysts indicate that MPs addition for lake

482 restoration would not endanger the long-term presence of *B. calyciflorus.*

483

484 **1. Conclusions**

485 In the algal growth test, no inhibition occurred at the two lowest MPs concentrations

486 (0.01 and 0.05 g l^{-1}). Considering the 53 mg MPs: mg P mass ratio as the adsorption

487 efficiency ratio (de Vicente et al., 2010; Merino-Martos et al., 2011), these

488 concentrations would match to P concentrations lower than 0.94 mg P l^{-1}, which are

489 much higher than typical concentrations found in natural waters (hypereutrophic

490 category correspond to annual mean TP concentration > 100 µg l^{-1}; Nürnberg, 1996).

491 Therefore, it is unlikely that MPs addition in a whole-lake application may cause

492 negative effect on algal growth. For higher MPs dose (EC_{50} for *Chlorella* sp. was 0.15 g

493 l^{-1}), no nutrient limitations but high turbidity ("shading effect") and Tot-Fe_{dis} values

494 cause negative effects on algal growth. In fact, algal growth inhibition was higher than

495 80% when adding 0.5 g MPs l^{-1} which lastly caused extreme turbidity values (>500

496 NTU). Additionally, Tot-Fe_{dis} concentration was significantly higher in 1.5 and 2 g MPs

497 l^{-1} treatments than in control. For the case of *B. calyciflorus*, LC_{50} was 1.63 g MPs l^{-1}

498   which is in the range of EC_{50} (immobilization; at 24 h) values reported for other

499   planktonic (1.99 g MPs l^{-1} for *D. magna*, Álvarez-Manzaneda et al., 2017) and benthic

500   organisms (1.57 g MPs l^{-1} for *Chironomus* sp.; Álvarez-Manzaneda et al., 2017). When

501   analyzing Tot-Fe$_{dis}$ effect on hatching rate, no significant effects were found (after 22 h,

502   the hatching of *B. calyciflorus* cysts was 100% for all treatments). Therefore, it is unlike

503   that the increase on Tot-Fe$_{dis}$ by MPs addition in a whole-lake application may cause

504   any negative effect on rotifer community. The high hatching rate is in accordance with

505   the extremely low Tot-Fe$_{dis}$ concentrations, ranging from 0 to 0.061 mg l^{-1}, which are

506   much lower than those recorded as negative for the survival of *B. calyciflorus*. To sum

507   up our results for *B. calyciflorus* lethal and sublethal toxicity tests, it is extremely

508   unlikely the mortality of adult organisms in contact with MPs (LC_{50} was 1.63 g MPs l^{-1}

509   which correspond to 30.7 mg P l^{-1}) as well as an affectation of the rotifer egg bank. In

510   conclusion, it is expected that MPs addition in a real whole-lake application cause

511   minor lethal and sublethal effects on both *Chlorella* sp. and *B. calyciflorus.* However,

512   further research for assessing MPs effects on lake biota is required. An outstanding

513   aspect is to study the effect of double exposition which may considerably reduce LC_{50}

514   values as well as MPs addition effects on ecological processes such as primary and

515   secondary production.

516

522

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838   **List of figures**

839   **Figure 1.** *Chlorella* sp. growth inhibition (%) as a function of MPs concentration.

840   Please note that EC_{50} is shown.

841   **Figure 2.** MPs effect on water turbidity (a) and on P-SRP concentration (b).

842   **Figure 3.** Dead organisms (%) of *B. calyciflorus* in contact, for 24 h, with MPs. Vertical

843   error bars show standard deviation of data (SD). n=9.

844   **Figure 4**. Temporal evolution of hatched cyts of *B. calyciflorus* (%) in the different

845   treatments. Nominal MPs concentrations are represented in different lines.

846   **Figure 5**. Dissolved iron concentration (Tot-Fe$_{dis}$) as a function of nominal MPs

847   concentration during the *B. calyciflorus* hatching experiment. Vertical error bars show

848   standard deviation of data (SD). Asterisk show significant differences (Kruskal Wallis

849   and U of Mann-Whitney test).
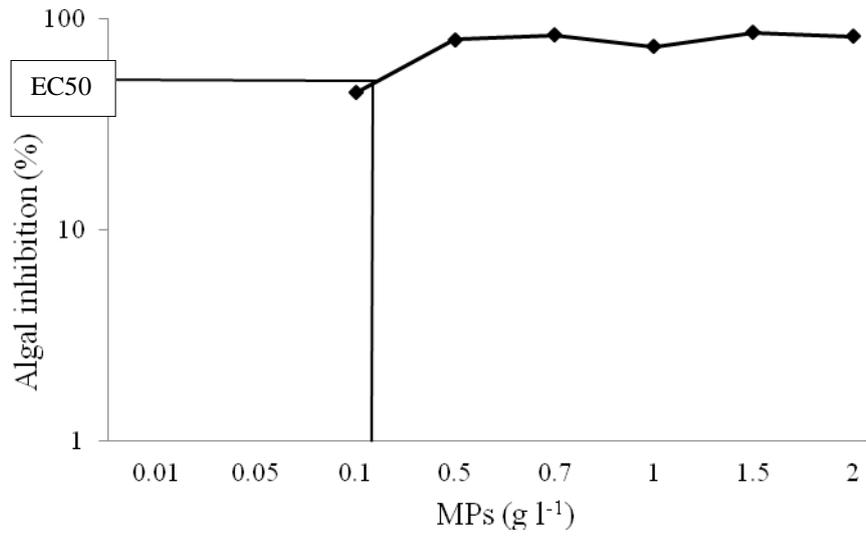
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868 **Figure 1**
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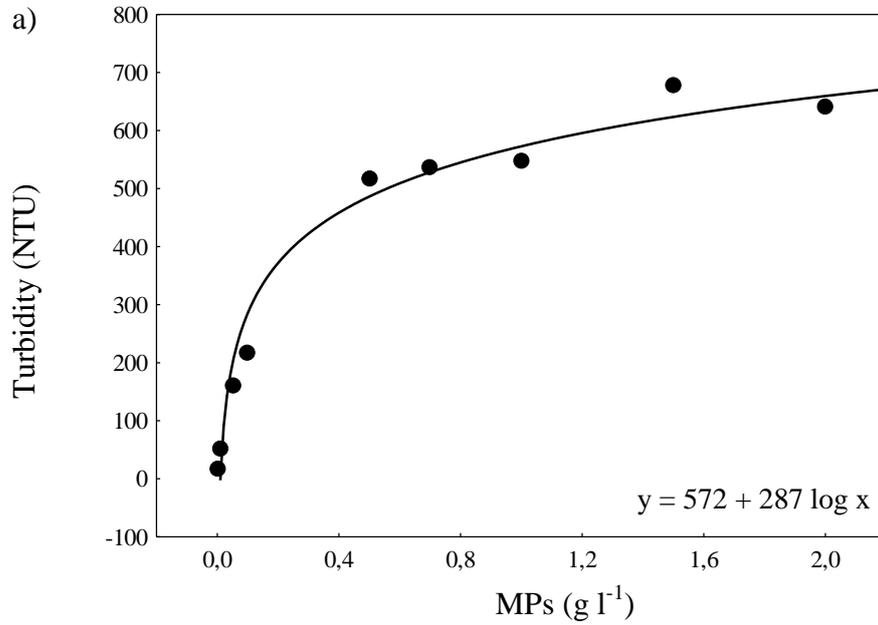
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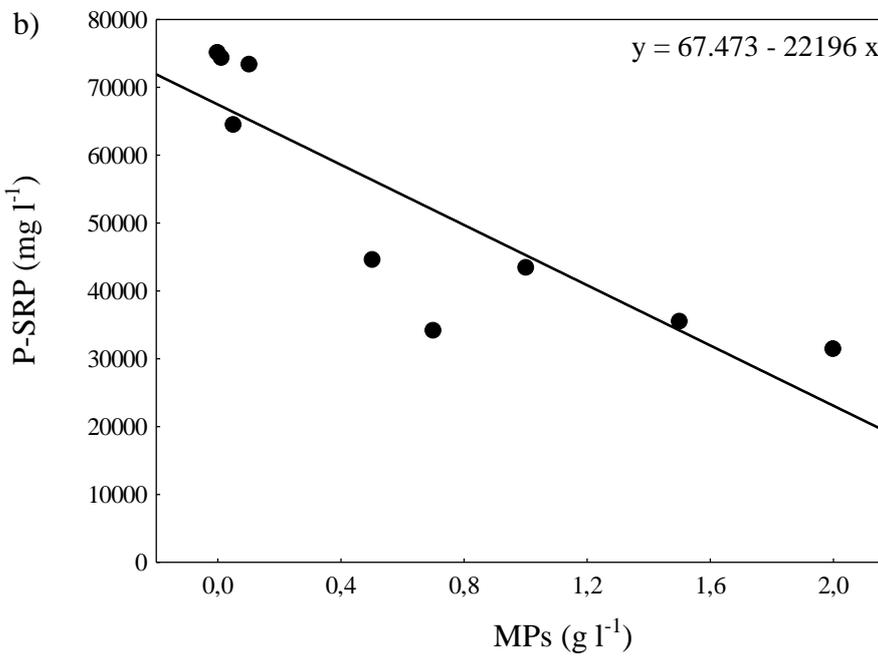915
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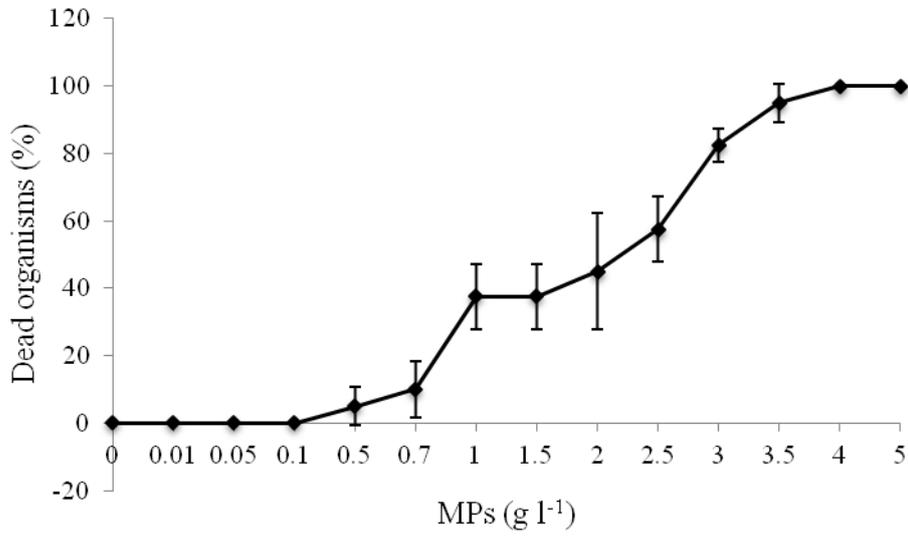a) $y = 572 + 287 \log x$

Turbidity (NTU)

MPs (g l^{-1})

b) $y = 67.473 - 22196 x$

P-SRP (mg l^{-1})

MPs (g l^{-1})

Figure 2

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940   **Figure 3**

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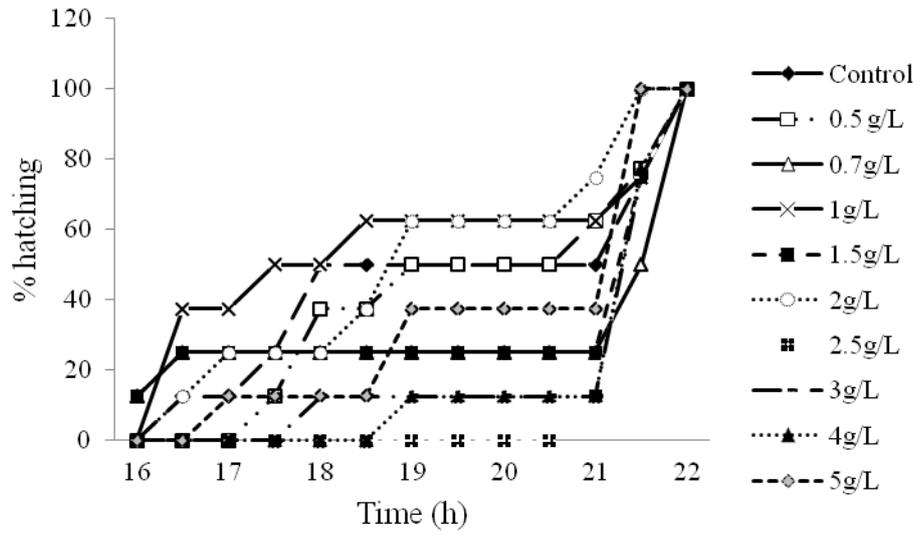
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964 **Figure 4**
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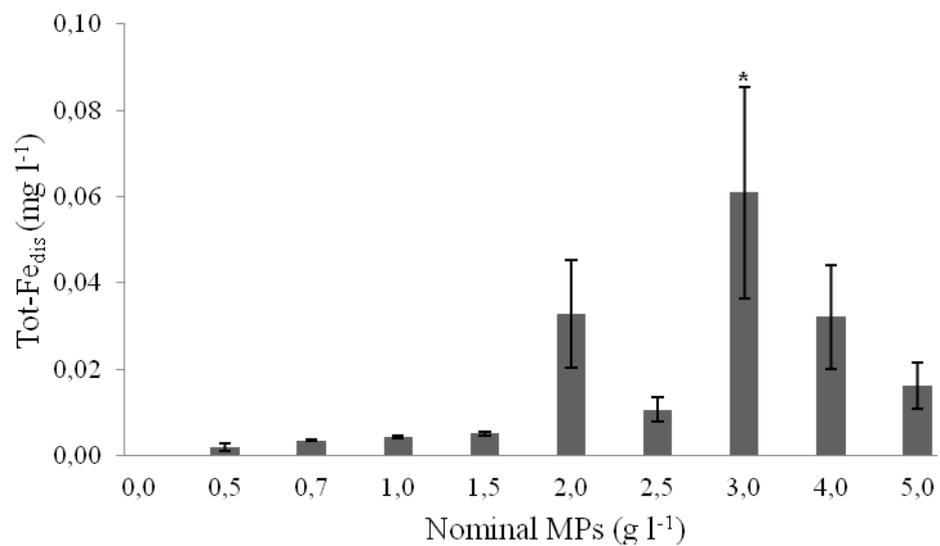979

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985 **Figure 5**
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Table 1. Physical-chemical parameters at the end of the *Chlorella* sp. growth inhibition

test (mean ± SD). Italics bold numbers show significant differences compared to

control. [1]Kruskal-Wallis ANOVA and [2]U of Mann-Whitney test.

MPs (g l^{-1})	[2]pH	[2]Conductivity (µS cm^{-1})	[1]O$_2$(mg l^{-1})	[2]Turbidity(NTU)
Control	5.78 ± 0.48	508 ± 7	7.8 ± 0.1	17.90 ± 9.77
0.01	6.35 ± 0.06	509 ± 18	7.9 ± 0.1	*53.15 ± 18.50*
0.05	*7.08 ± 0.30*	541.25 ± 22	7.7 ± 0.3	*160.75 ± 118.11*
0.1	*6.85 ± 0.30*	557 ± 35	7.7 ± 0.1	*216.47 ± 189.87*
0.5	*7.39 ± 0.28*	577.5 ± 47	7.8 ± 0.1	*517.00 ± 412.48*
0.7	*7.52 ± 0.19*	540 ± 17	7.7 ± 0.1	*536.50 ± 437.00*
1	*7.44 ± 0.35*	*606 ± 9*	7.7 ± 0.0	*548.50 ± 431.38*
1.5	*7.67 ± 0.20*	597.5 ± 69	7.5 ± 0.4	*677.50 ± 287.20*
2	*7.89 ± 0.30*	*583 ± 161*	7.7 ± 0.1	*641.75 ± 345.59*

1024 **Table 2.** Chemical parameters measured at the end of the *Chlorella* sp. growth

1025 inhibition test (mean ± SD). Italics bold numbers show significant differences in

1026 compared to control. [1]Kruskal-Wallis ANOVA and [2]U of Mann-Whitney test.

MPs (g l^{-1})	[1]N-NH$_4^+$ (mg l^{-1})	[2]N-NO$_3^-$ (mg l^{-1})	[2]N-NO$_2^-$ (mg l^{-1})	[2]P-PO$_4^{3-}$ (mg l^{-1})	[2]Tot-Fe$_{dis}$ (mg l^{-1})	[2]Growth inhibition/Control (%)
Control	0.26 ± 0.51	105.46 ± 35.10	0.02 ± 0.02	75132.40 ± 24000.75	1.11 ± 0.11	
0.01	0.00 ± 0.00	114.34 ± 31.25	*0.00 ± 0.00*	74311.39 ± 16447.17	1.59 ± 0.08	0
0.05	0.00 ± 0.00	107.09 ± 21.17	0.64 ± 0.71	64551.76 ± 5297.70	1.85 ± 0.10	0
0.1	0.02 ± 0.11	132.19 ± 17.38	*0.4 ± 0.16*	73500.80 ± 12020.38	2.44 ± 0.47	44.9 ± 51.4
0.5	0.03 ± 0.10	145.10 ± 7.78	*1.27 ± 1.35*	44729.53 ± 20953.80	11.11 ± 6.29	*80.3 ± 15.3*
0.7	0.02 ± 0.09	*150.84 ± 12.68*	*1.39 ± 1.22*	34220.57 ± 21573.58	12.65 ± 3.24	*84.1 ± 8.7*
1	0.18 ± 0.26	147.30 ± 11.49	*1.59 ± 1.3*	43539.06 ± *12934.86*	16.94 ± 7.77	*74.2 ± 29.2*
1.5	0.12 ± 0.22	*154.32 ± 10.8*	*1.78 ± 0.95*	*35657.35 ± 15225.11*	*30.24 ± 6.64*	*86.6 ± 29.2*
2	0.09 ± 0.16	*144.49 ± 7.44*	*1.67 ± 1.08*	*31552.28 ± 11920.22*	*22.62 ± 2.57*	*82.8 ± 19.2*

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1039 **Table 3.** Physical-chemical parameters at the beginning (T_0) and at the end (T_f);

1040 synchronized time (Ts) and Time of the First Hatching (TFH) of the experiment with *B.*

1041 *calyciflorus* (mean ± SD).

	T (ºC)	pH	Hardness	O_2 (%)	Ts (h)	TFH (h)
Control						
T_0	21.07	10.45	2.33	83.56	5.2	16.5
T_f	22.9	10.36	2.33			
Treatments						
T_0	21.11 ± 0.09	10.37 ± 0.04	2.35 ± 0.02	83.61 ± 0.14	4.2± 1.6	17.5 ±1.6
T_f	22.82 ± 0.07	10.37 ± 0.03	2.30 ± 0.02			

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