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1 Improved short-term toxicity test protocol to assess metal tolerance in phototrophic periphyton:  
2 Toward standardization of PICT approaches

3

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28 Abstract

29 Pollution-induced community tolerance (PICT) approaches involve comparing tolerance levels of  
30 natural communities to a particular contaminant or a contaminant mixture using short-term toxicity  
31 tests performed under controlled conditions. However, results from toxicity tests can be modulated by  
32 various environmental and experimental conditions, raising questions about their reproducibility and  
33 comparability. In this context, the present study aimed to determine the influence of exposure  
34 duration, periphyton suspension concentration and periphyton maturation stage on the measurement of  
35 short-term effects of copper on phototrophic periphyton communities. Our results showed the very  
36 weak influence of exposure duration in the tested range (2–6 h) on toxicity level, whereas periphyton  
37 biomass in the tested suspension (in terms of both chlorophyll *a* concentrations and dry weight),  
38 proved a crucial determinant in toxicity assessment. Results also highlighted the potential tolerance  
39 increase with the periphyton maturation stage. This parameter conditioned the positive linear  
40 relationship between tolerance level and periphyton suspension concentration, leading to an increase  
41 in the linear regression slope with the maturation stage. This suggests that such a relationship is  
42 probably highly periphyton-dependent. Consequently, to enable data toxicity comparisons, an *a priori*  
43 normalization of the periphyton suspension biomass is necessary, and PICT approaches require the  
44 use, as much of possible, of periphyton with similar maturation stage. Finally, the present study clearly  
45 shows that a better standardization of PICT approaches could help to improve reproducibility. It could  
46 thus facilitate the comparison of tolerance levels measured in the same study (e.g. spatial and/or  
47 temporal and/or inter-treatment comparison) as well as the comparison obtained from different  
48 experimental and *in situ* research.

49

50 Keywords

51 Microbial ecotoxicology; Biofilm; PICT; Short-term toxicity tests; Metal.

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## 55           **1 Introduction**

56   The concept of pollution-induced community tolerance (PICT), first introduced by Blanck et al  
57   (1988), is an ecotoxicological tool that can help to characterize environmental status, since it takes into  
58   account the contamination history of the ecosystem at the community level (Tlili and Montuelle 2011).  
59   PICT is based on the fact that chronic exposure of a biological community to a toxicant will induce a  
60   succession of species resulting in a replacement of the sensitive species by more tolerant ones (Blanck  
61   2002) and/or specific adaptation such as the establishment of mechanisms for detoxification, thus  
62   leading to an increased community tolerance to this toxicant (Tlili and Montuelle 2011). The approach  
63   uses the theoretical basis of toxicology (the dose-response model) to quantify community tolerance  
64   (Schmitt-Jansen et al. 2008), and can give an indication of past *in situ* exposure of the community to  
65   toxicants (Tlili and Montuelle 2011).

66   In principle, the PICT concept can be applied to terrestrial and aquatic ecosystems (Hjorth et al. 2006;  
67   Niklinska et al. 2006). Although, they can be applied with vertebrates (Knopper and Siciliano 2002)  
68   and invertebrates (Millward and Grant 2000), PICT approaches are used mainly for microbial  
69   communities such as bacteria (Boivin et al. 2005) and photosynthetic microorganisms (Dahl and  
70   Blanck 1996). Induced tolerance is observed by comparing the sensitivity of natural communities to a  
71   particular contaminant or contaminant mixture (Kim-Tiam et al. 2014), using short-term toxicity tests  
72   performed under controlled conditions. These tests are based on representative endpoints such as,  
73   photosynthetic efficiency (Dorigo et al. 2010; Tlili et al. 2010), respiration potential (Dorigo et al.  
74   2010; Tlili et al. 2011) enzymatic activities (Fechner et al. 2012), and metabolic capacities (Boivin et  
75   al. 2005). Different studies have thus highlighted an increased tolerance of microbial communities to  
76   various substances such as metals and metalloids, and organic pesticides following chronic exposure  
77   to these toxicants in various ecosystems (see for reviews Blanck 2002; Imfeld and Vuilleumier 2012;  
78   Tlili and Montuelle 2011). In aquatic environments, metals including copper, zinc, lead, nickel,  
79   mercury, and cadmium have been particularly well-studied in PICT approaches, because of their  
80   abundance in surface waters and their potential toxicity to aquatic microbial communities (e.g.  
81   Gustavson et al. 1999; Soldo and Berha 2000; Barranguet et al. 2002; Serra and Guasch 2009).

82 In lotic environments, the PICT approach is frequently applied on surface-associated microbial  
83 communities, called periphyton (or biofilm). Periphyton is a complex assembly of microbial  
84 communities (including microalgae, bacteria, fungi and heterotrophic protists) embedded in a  
85 polysaccharide and protein matrix. Since they are attached to natural surfaces, periphyton integrate the  
86 effects of multiple anthropogenic disturbances over extended periods of time, and have strong  
87 capacities to adapt to environmental changes (Sabater et al. 2007). Tolerance assessment of  
88 phototrophic periphyton communities (mainly composed of microalgae and cynaobacteria) is  
89 generally performed using photosynthesis as a functional parameter (Dahl and Blanck 1996; Bérard et  
90 al. 2003).

91 However, short-term effects of metals on photosynthesis can be modulated by various environmental  
92 and experimental conditions, and protocols used in toxicity test vary widely among studies, sometimes  
93 hindering comparison of results (Eklund and Kautsky 2003). For example, it has been shown that  
94 metal effects on microalgae depend not only on metal concentration, but also on cellular densities  
95 (Tobar et al. 1993). This biological parameter can modulate accumulation of metal on individual cells,  
96 thus modulating their exposure level (Steemann and Wium-Andersen 1970; Moreno-Garrido 1997).  
97 Moreno-Garrido et al. (2000) thus observed an increase in copper toxicity (i.e. a decrease in half  
98 effective concentration ( $EC_{50}$ ) values) to marine microalgal species when their initial cellular density  
99 decreased. In the same way, Fechner et al. (2010) observed for heterotrophic periphyton communities  
100 an increase in metals  $EC_{50}$  values concomitantly with an increase in periphyton suspension  
101 concentrations (periphyton biomass used during the toxicity test). The literature reveals that exposure  
102 durations in toxicity tests with metals can vary widely among studies. For example, in the case of  
103 short-term toxicity tests to evaluate effects of copper on photosynthetic efficiency of periphyton,  
104 exposure duration (incubation duration) can be 2 h (Tlili et al. 2010; Lambert et al. 2012), 4 h (Guasch  
105 et al. 2004) or 6 h (Serra and Guasch 2009), but the influence of such differences on the obtained  
106 results is still sparsely documented. Since these parameters are rarely taken into account (or described)  
107 in studies using short-term toxicity tests, prior findings confirm the need for better standardization of  
108 toxicity tests for reproducibility and comparability of toxicity data, as previously stated (Soldo and

109 Berha 2000; Eklund and Kautsky 2003; Fechner et al. 2010). It is now well accepted that PICT can  
110 serve as a powerful tool for *in situ* assessment of chronic effects of toxicants on periphyton  
111 communities (Pesce et al. 2010). This kind of approach raises question about the periphyton sampling  
112 procedure, which appears to be highly variable between studies, depending on the use of either natural  
113 (Dorigo et al. 2010) or artificial substrates, with fluctuating colonization durations (e.g from 2 weeks  
114 (Blanck and Dahl 1996) to 2 months (Pesce et al. 2010)). This notably raises the question of the  
115 influence of the maturation stage (growth stage) in tolerance assessment. Maturation processes include  
116 both species succession, which conditions the tolerance baseline of the community, and the building of  
117 a highly complicated structure that can form a protective layer (Extracellular Polymeric Substances),  
118 thereby influencing the level of exposure to toxicants (Ivorra et al. 2000; Gold et al. 2003a, 2003b).  
119 Given this background, the main aim of our work was to help identify the most important parameters  
120 that must be considered to obtain reproducible and comparable results from short-term toxicity tests  
121 for metals on natural phototrophic periphyton communities.

122 Accordingly, we developed a microcosm approach aiming to accurately determine the influence of  
123 three main parameters on the significance and reproducibility of results obtained from short-term  
124 dose-response relationships for metals (as performed for tolerance determination in the PICT detection  
125 step). Copper was chosen as model compound because of its frequent occurrence in freshwater  
126 environments (Fechner et al. 2012) as well as its current use in PICT approaches. We thus focused on  
127 the influence of (i) periphyton suspension concentration, (ii) duration of exposure to copper, and (iii)  
128 periphyton maturation stage.

129

## 130 **2. Materials and Methods**

### 131 **2.1. Experimental setup and periphyton sampling procedure**

132 Microcosm experiments were carried out in three independent microcosms (glass, 40 × 20 × 25 cm),  
133 incubated in thermoregulated tanks at 23 °C (polyethylene, 250 L, 121 × 81 × 33cm). One submersible  
134 pump (New Jet 800) was introduced in each microcosm to reproduce the water flow (water discharge

135 ~1.2 L/min), and three pumps were placed in the tank to homogenize water temperature. High-  
136 pressure sodium lamps were used to obtain a constant light intensity of 3500 lux ( $42.7 \mu\text{mol m}^{-2} \text{s}^{-1}$ )  
137 under a 13 h/11 h light/dark photoperiod. Artificial substrates (glass slides) were previously placed  
138 vertically at the surface in each microcosm to allow periphyton settlement. Microcosms were filled  
139 with untreated groundwater and a suspension of natural periphyton, collected on stones in April 2013  
140 at the upstream reference site of the Morcille River (Beaujolais, Eastern France) with low Cu  
141 concentrations (dissolved concentrations lower than 0.9  $\mu\text{g/L}$ ; see Montuelle et al. (2010) for details).  
142 Silica, nitrates and phosphates were added at concentrations of 15 mg/L, 8 mg/L, and 0.2 mg/L,  
143 respectively, to reach sampling site concentrations (Dorigo et al., 2010). Water was renewed weekly to  
144 avoid nutrient depletion. The experiment lasted 5 weeks. In order to obtain a periphyton suspension  
145 for short-term bioassays, two series of samples were taken from the microcosms, after 3 and 5 weeks  
146 of colonization, respectively. The periphyton was scraped from glass substrates collected in each  
147 microcosm, and suspended in a diluted equivalent mixture of demineralized water and mineral water  
148 (Evian, France) to obtain three replicates of periphyton suspension at 36  $\text{cm}^2/\text{mL}$ .

149

## 150 2.2. Short-term bioassays

151 The same medium should be used for all toxicity tests, in order to avoid changes in metal  
152 bioavailability during metal toxicity testing (Blanck 2002, Blanck et al. 2003). Accordingly and to  
153 improve standardization an equivalent mixture of demineralized water and mineral water (Evian,  
154 France) was therefore used to prepare periphyton suspensions as well as Cu solutions. A semi-  
155 logarithmic series of Cu concentrations was freshly prepared to obtain five Cu concentrations, ranging  
156 from about 0.32 to 32 mg/L. Cu concentrations in each dilution were checked using inductively  
157 coupled plasma-mass spectrometry (ICP-MS X Series II, Thermo Electron).

158 The tolerance of phototrophic periphyton communities to Cu was assessed using photosynthetic  
159 efficiency as endpoint. Photosynthetic efficiency is based on the measurement of maximal quantum  
160 yield ( $Y_{II}$ ) of algae (Schreiber et al. 2002). It reflects the number of functional photosystems II (PSII),  
161 and therefore more generally the physiological state of the phototrophic communities (Tlili et al.

162 2010). To assess this parameter, periphyton suspensions (1.8 mL, see procedure below) were exposed  
163 to increasing concentrations of Cu (0.9 mL) in a climatic chamber at 23 °C (temperature of  
164 microcosms during growth period) (MLR-350 Versatile Environmental Test Chamber, Sanyo) under  
165 artificial light (1400 lux). Samples were then kept for 30 min in a dark chamber and PSII quantum  
166 yield (665 nm) was determined using a PhytoPAM (pulse amplitude-modulated) fluorometer (Heinz  
167 Wals, GmbH). For each sample replicate, four blanks and two analytical replicates were analyzed for  
168 each concentration. A single saturation pulse was applied to calculate the maximal quantum yield as  
169  $Y_{II, 665\text{ nm}} = (F_m - F_0)/F_m$ , where  $F_m$  is the maximum fluorescence after the saturation pulse, and  $F_0$  is  
170 the steady state fluorescence.

171 Three variable parameters were assessed during the short-term toxicity tests: (i) periphyton biomass,  
172 (ii) exposure duration, and (ii) maturation stage. Given the predominance of phototrophic  
173 microorganisms in illuminated periphyton, we postulated that the estimation of the phototrophic  
174 biomass (based on *chl a* concentrations) might give a relative estimation of the total biomass. The  
175 influence of periphyton biomass was thus tested using various periphyton suspension concentrations.  
176 Successive dilutions of the initial periphyton suspension were performed with the  
177 demineralized/mineral water mixture (see above) to obtain periphyton suspension concentrations  
178 ranging from about 400 µg *chl a*/L (dilution 4, i.e. D4) to about 7000 µg *chl a*/L (D1). These  
179 concentrations were estimated from chlorophylla (*chl a*) measurement with the PhytoPAM  
180 fluorometer. This relative estimation of periphyton suspension biomass was then checked with 5-week  
181 periphyton by measuring total biomass (dry weight, DW) as described in Morin et al. (2010).

182 The influence of the duration of exposure to Cu was evaluated from toxicity tests after 2 h, 4 h and 6 h  
183 of incubation. The influence of the periphyton maturation stage was assessed by performing toxicity  
184 tests with periphyton retrieved after 3 and 5 weeks of development in the microcosms.

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## 189 2.3. Statistical analysis

190 The relationship between *chl a* and periphyton DW was assessed using linear regression analysis. The  
191 analysis of results from short-term bioassay was conducted using functions from the ‘drc’ package  
192 (Ritz and Streibig 2005) in R version 2.15.0 (R Development Core Team 2012). Dose-response curves  
193 were fitted to the data using the four-parameter log-logistic model given by the formula:

$$194 \text{ response} = c + \frac{d - c}{1 + \exp\{b \times (\log(Dose) - \log(e))\}}$$

195 where *b* denotes the slope of the curve around *e*. Parameters *c* and *d* are the lower and upper limits of  
196 the curve, respectively, and *e* denotes the EC<sub>50</sub>, the dose producing a response half-way between the  
197 upper and the lower limit. The response variable was expressed as the percentage of the yield of  
198 uncontaminated control assay. In addition, EC<sub>10</sub>, and EC<sub>50</sub> were derived from each dose-response  
199 curve. Data from the three replicate bioassays were pooled to produce a single dose-response curve  
200 with the % inhibition of PSII activity as a function of Cu concentrations. Student *t* tests were used to  
201 determine significant differences in EC<sub>*x*</sub> between treatments using the selectivity index (SI) function  
202 from the ‘drc’ package. Linear regression analyses were performed to evaluate the relationship  
203 between EC<sub>*x*</sub> and *chl a* concentrations. Analysis of covariance (ANCOVA) was used to determine  
204 whether the relationship differed between periphyton maturation stages.

205

## 206 3. Results and discussion

### 207 3.1. Influence of tested parameters

208 Assessment of short-term toxicity of Cu to 3-week periphyton photosynthetic efficiency showed a  
209 positive correlation between EC<sub>*x*</sub> and periphyton suspension concentration, whatever the exposure  
210 duration (Table 1; Fig. 2). For example, after 6 h of incubation, EC<sub>50</sub> increased about 14-fold between  
211 the most diluted periphyton suspension (D4, mean EC<sub>50</sub> = 0.09 mg/L) and the least (D1, mean  
212 EC<sub>50</sub> = 1.23 mg/L), which was characterized by *chl a* concentrations (6980 µg/L) about 17 times

213 higher than *chl a* concentrations in D4 samples (420 µg/L). Also, the positive linear relationship  
214 ( $p < 0.05$ ) was observed between *chl a* concentrations in periphyton suspension and both  $EC_{50}$  and  
215  $EC_{10}$  values (Fig. 2), whatever the periphyton maturation stage (3 and 5 weeks). A significant linear  
216 relationship ( $p < 0.05$ ) was also obtained when comparing  $EC_x$  and total biomass (based on DW  
217 measurement), which was determined for the 5-week periphyton (data not shown). These results thus  
218 highlight the marked influence of the periphyton suspension concentration on tolerance measurement  
219 in toxicity tests. Performing short-term toxicity tests based on  $\beta$ -glucosidase enzymatic activity,  
220 Fechner et al. (2010) also observed an increase in  $EC_{50}$  values with an increase in concentration of  
221 periphyton suspensions. Ahuja et al. (1999) showed that increasing biomass concentration from 0.04  
222 to 0.20 gDW/L decreased the level of metal binding per unit cell mass. In addition, according to  
223 Monteiro and Castro (2012), the effective surface area available for sorption, and the average distance  
224 between available adsorption sites, is reduced by partial aggregation of biomass at high biomass.  
225 These studies therefore suggest that the observed increase in tolerance to Cu at higher periphyton  
226 suspension concentration in the present study is due, at least partially, to a decrease in periphyton  
227 exposure to Cu during toxicity testing.

228 We also tested, with a 3-week periphyton, and for the various periphyton suspension concentrations,  
229 the influence of exposure duration (2, 4 and 6 h) to Cu on the tolerance measurement (Table1).  
230 Despite the increase in  $EC_{50}$  values with the increase in periphyton suspension concentration used in  
231 the toxicity test, no significant difference was observed for this parameter between the three tested  
232 exposure durations (2 h, 4 h and 6 h of incubation), whatever the periphyton dilution (D1 to D4).  
233 Effects of exposure duration on  $EC_{10}$  were also imperceptible or very limited, the only significant  
234 differences being observed between 4 h and 6 h of incubation for the periphyton suspension  
235 concentration D3, with values of the same order of magnitude (0.01 to 0.05 mg/L). Within the range of  
236 test conditions in the present study (2–6 h), exposure duration had a negligible influence on the  
237 measurement of tolerance to Cu whatever the periphyton suspension concentrations. Tlili et al (2011)  
238 also observed that the dose-response curve did not change with longer exposure to copper (3, 4 and  
239 6h) when performing toxicity tests based on respiration, and they opted for the shorter exposure time.

240 In our toxicity tests based on photosynthetic efficiency, a 2 h incubation seems sufficient for the  
241 cellular uptake of Cu. Thus we decided to perform the following short-term toxicity tests (toxicity test  
242 with a 5-week periphyton) using 2 h of incubation as previously used by Tlili et al. (2010).

243 The third parameter tested was the influence of the maturation stage of the periphyton.  $EC_{50}$  and  $EC_{10}$   
244 were determined from short-term toxicity tests, after 2 h of incubation, performed with 3- and 5-week  
245 periphyton (Fig 2), considering different periphyton suspension concentrations (D4 to D1). At equal  
246 periphyton suspension concentration, and despite the variability between replicates,  $EC_x$  mean values  
247 were higher for 5-week periphyton than for 3-week periphyton ( $EC_{50}$ : D3  $p < 0.05$ ;  $EC_{10}$ : D1, D3  
248  $p < 0.05$ , Fig 2).

249 In the present study, phototrophic periphyton had a greater tolerance to Cu at higher maturation stage,  
250 highlighting the potential variability of tolerance levels with periphyton maturation stage during  
251 toxicity tests. This result is consistent with the study of Ivorra et al. (2000), who showed a variation in  
252 the effects of metal exposure (Zn and Cd) with the developmental stage of the periphyton. They  
253 observed that mature periphyton were more resistant to metals, even without a history of pre-exposure.  
254 A resistance to Cd was also observed on mature phototrophic periphyton by Duong et al. (2010).  
255 Studying Cu effects, Tien and Chen (2013) explained the decreased metal accumulation in a more  
256 mature periphyton by a decrease in metal-binding capacity thus probably reducing the exposure of  
257 periphyton communities. These results could explain our findings concerning the increased tolerance  
258 to Cu at higher periphyton maturation stage during toxicity tests. In addition, although the relationship  
259 between  $EC_x$  values and *chl a* concentrations (used as a proxy of periphyton suspension concentration  
260 for the toxicity test) was significant ( $p < 0.05$ ) whatever the age of the periphyton, it was modulated  
261 according to the sample considered (3 or 5 weeks; Fig. 1) as shown by the significant difference in  
262 slope values ( $p < 0.05$ ). The slope value of the linear relationship obtained with a 5 weeks periphyton  
263 was significantly higher than that obtained with a 3-week periphyton ( $p < 0.05$ ), thus suggesting that at  
264 higher biomass, the protective effect is stronger for a periphyton at a later maturation stage.

265

266

267 3.2. Standardization perspectives

268 The results of this study argue for more standardized procedures adapted to PICT approaches. Among  
269 the different parameters tested, exposure duration seems to have the least influence on tolerance  
270 measurement. However, short-term toxicity tests are generally performed in small water volumes with  
271 limited periphyton quantities, which can quickly induce significant changes in microbial community  
272 because of the "bottle effect". This was first observed by Schelske (1984), who showed that effects of  
273 confinement on the structure of phytoplankton communities could outweigh toxicant effects.  
274 However, PICT is based on the fact that a chronic exposure of communities to a toxicant can induce  
275 shift in species composition, thus leading to an increase in tolerance capacities. Indeed, it is better to  
276 limit as much as possible community changes during toxicity testing (i.e. PICT detection).  
277 Accordingly, we recommend short exposure (i.e. 2 h). Based on literature analysis, this duration seems  
278 sufficient to assess short-term effects of other metals, such as zinc, nickel and silver, on phototrophic  
279 periphyton (Soldo and Behra 2000)

280 By contrast, we showed that periphyton biomass in the tested suspension (in terms of both *chl a*  
281 concentrations and DW) is a crucial determinant in toxicity assessment because of its significant  
282 influence on tolerance level. Consequently, this parameter should be standardized to obtain  
283 comparable results between studies. Previous authors suggested using an *a posteriori* normalization of  
284 toxicity data, based on periphyton biomass (Soldo and Behra 2000; Fechner et al. 2010; Lambert et al.  
285 2012). In these studies, toxicity data were normalized by dividing  $EC_x$  by the periphyton dry weight  
286 (expressed in grams of dry matter per liter) (Fechner et al. 2010) or by the *chl a* concentration in the  
287 periphyton suspension (Soldo and Behra 2000; Lambert et al. 2012). However, our results revealed  
288 that the linear relationship observed between  $EC_x$  and periphyton suspension concentration (expressed  
289 as *chl a* or DW) depends on periphyton maturation stage, as shown by the significant difference in the  
290 slopes of the relationship observed respectively with 3-week and 5-week periphyton (ANCOVA  
291  $p < 0.05$ , Fig 1). Accordingly, an *a posteriori* normalization could sometimes be inappropriate since  
292 periphyton structure continuously evolves, and can differ widely according to numerous biotic and  
293 abiotic parameters. More generally, and independently of the importance of the maturation stage, our

294 results suggest that the relationship between  $EC_x$  and *chl a* is probably highly periphyton-dependent.  
295 Hence in order to take into account the differences between sampling sites or between sampling  
296 periods and thus enable data toxicity comparisons, an *a priori* normalization of the periphyton  
297 suspension biomass is advocated.  
298 However, one challenge of such an *a priori* normalization resides in the fact that toxicity tests must be  
299 performed with fresh periphyton, rapidly after sample collection. As DW measurement is time-  
300 consuming because of methodological constraints, the use of *chl a* concentrations, as a proxy of  
301 biomass, could be a good alternative, because of the possible use of PhytoPam (or other fluorimetric  
302 measurement), which allows an immediate estimation of *chl a* concentrations. In the present study, this  
303 alternative appears methodologically robust as shown by the close positive linear relationship between  
304 DW and *chl a* obtained in the various dilutions performed with the 5-week periphyton ( $p < 0.001$ ,  
305 Fig. 3). Hence it would be of interest to check whether this relationship between DW and *chl a* extends  
306 to periphyton with higher abundances of heterotrophic microorganisms. It can be argued that such a  
307 *a priori* normalization would require using disrupted periphyton (suspension periphyton). The use of  
308 disrupted or intact periphyton in short-term toxicity assessment is a recurrent debate, and it is known  
309 that the periphyton structure (i.e. suspended or attached) influences response to acute metal exposure.  
310 Given the protective role of the periphyton matrix, which limits the penetration of toxicants,  
311 undisrupted periphyton communities are generally more tolerant to metals than suspended periphyton  
312 communities (Barranguet et al. 2000; Guasch et al. 2003). Nevertheless, the high resistance of thick  
313 periphyton to metals can be mainly related to the physical structure of the assemblage, independently  
314 of species composition and their intrinsic tolerance properties (Barranguet et al. 2000). This can  
315 introduce a potential source of bias in PICT approaches which aim at evaluating changes in tolerance  
316 at the community level. Furthermore, since *a priori* normalization of periphyton biomass on attached  
317 assemblages appears impossible, our results offer new arguments in support of using disrupted  
318 periphyton, as is the case in many PICT studies (e.g. Soldo and Behra 2000; Dorigo et al. 2010; Tlili et  
319 al. 2010). According to the  $EC_x$  values obtained, it seems preferable to consider low periphyton  
320 biomass ( $chl a < 3000 \mu\text{g/L}$  or  $DW < 0.4 \text{ mg/mL}$  in our case) in order to reduce the variability

321 observed between replicates, because of the difficulty in homogenizing disrupting periphyton samples  
322 at high suspension concentrations.

323 We also confirmed in the present study the importance of the maturation stage of the periphyton used  
324 for toxicity tests. This raises particular concerns when using PICT approaches in natural environments  
325 with periphyton collected on natural substrates, without knowledge about the colonization duration.  
326 One possible alternative is the use of artificial substrates, as chosen by many authors (Guasch et al.  
327 2003; Dorigo et al. 2010; Morin et al. 2010), despite the well-known biases due to the fact that  
328 artificial substrates do not perfectly reflect natural substrates (Peterson et al. 1989; Potapova et al.  
329 2005). This alternative is of particular interest in PICT studies designed to assess spatial and/or  
330 temporal changes in tolerance. In this context, we note that it could be of interest to define the  
331 recommended colonization time before a periphyton can be used in toxicity tests. However, and given  
332 the fact that periphyton maturation greatly depends on various environmental factors and is strongly  
333 site-dependent, this parameter is probably one of the most difficult to define with a view to a better  
334 standardization of PICT approaches.

335 In addition to parameters considered in our study, it would be of interest to further identify and test  
336 other factors that could significantly influence results and conclusions from PICT studies (e.g.  
337 temperature incubation or light intensity).

338

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|           |     | <b>EC<sub>50</sub></b> | <b>EC<sub>10</sub></b> |
|-----------|-----|------------------------|------------------------|
| <b>D4</b> | 2 h | 0.21 ± 0.07            | 0.02 ± 0.02            |
|           | 4 h | 0.08 ± 0.02            | 0.01 ± 0.01            |
|           | 6 h | 0.09 ± 0.02            | 0.02 ± 0.02            |
| <b>D3</b> | 2 h | 0.11 ± 0.02            | 0.03 ± 0.02 (ab)       |
|           | 4 h | 0.10 ± 0.03            | 0.01 ± 0.01 (b)        |
|           | 6 h | 0.13 ± 0.02            | 0.05 ± 0.02 (a)        |
| <b>D2</b> | 2 h | 0.27 ± 0.05            | 0.11 ± 0.05            |
|           | 4 h | 0.30 ± 0.09            | 0.05 ± 0.02            |
|           | 6 h | 0.30 ± 0.06            | 0.06 ± 0.02            |
| <b>D1</b> | 2 h | 0.76 ± 0.24            | 0.13 ± 0.06            |
|           | 4 h | 0.92 ± 0.31            | 0.18 ± 0.09            |
|           | 6 h | 1.23 ± 0.52            | 0.19 ± 0.08            |

484

485 **Table 1.** Mean (± s.d.) EC<sub>50</sub> and EC<sub>10</sub> (mg Cu/L) for periphyton suspension concentrations, ranging from about  
 486 400 µg chl<sub>a</sub>/L (D4) to about 7000 µg chl<sub>a</sub>/L (D1) after 2, 4 and 6 h of incubation. Different letters (a, b, c)  
 487 indicate significant difference between EC<sub>10</sub> values ( $p < 0.05$ ),  $n = 3$ , within the D3 dilution.

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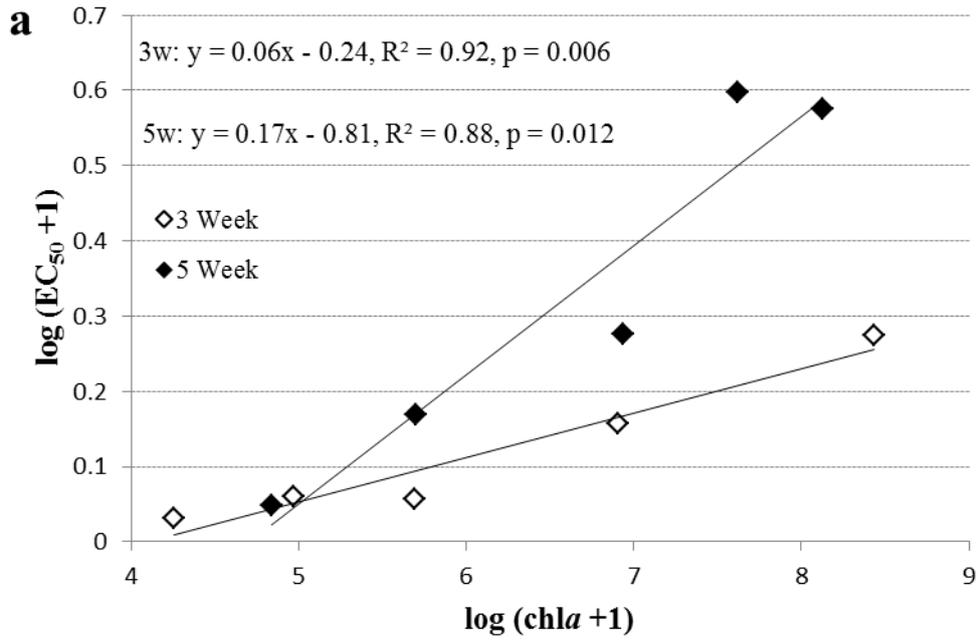
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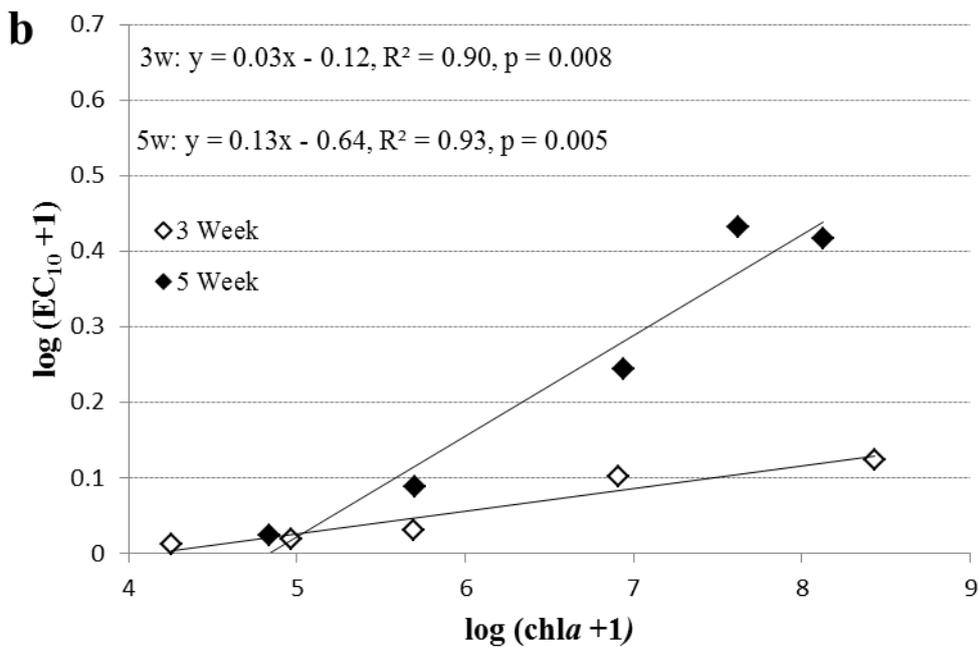
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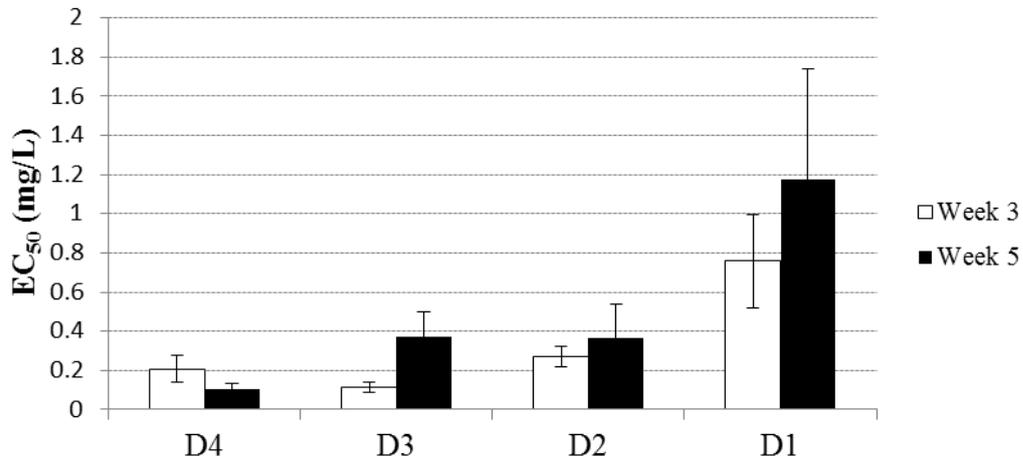


504

505 **Fig 1.** Linear relationship between mean *chla* concentrations in periphyton suspension ( $\mu\text{g/L}$ ) and (a) mean  $\text{EC}_{50}$   
506 and (b) mean  $\text{EC}_{10}$  (mg Cu/L) during toxicity tests performed with a 3- and 5-week periphyton after 2 h of  
507 incubation.

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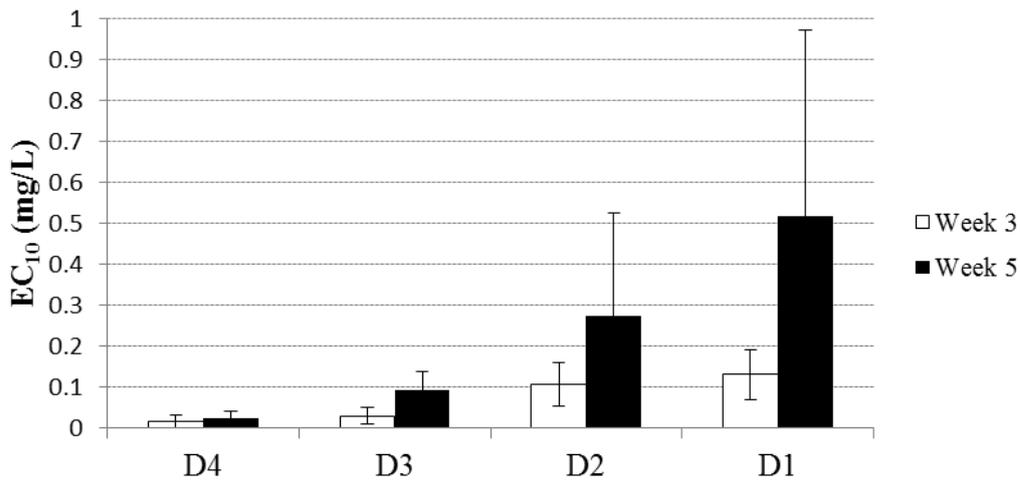
**a**



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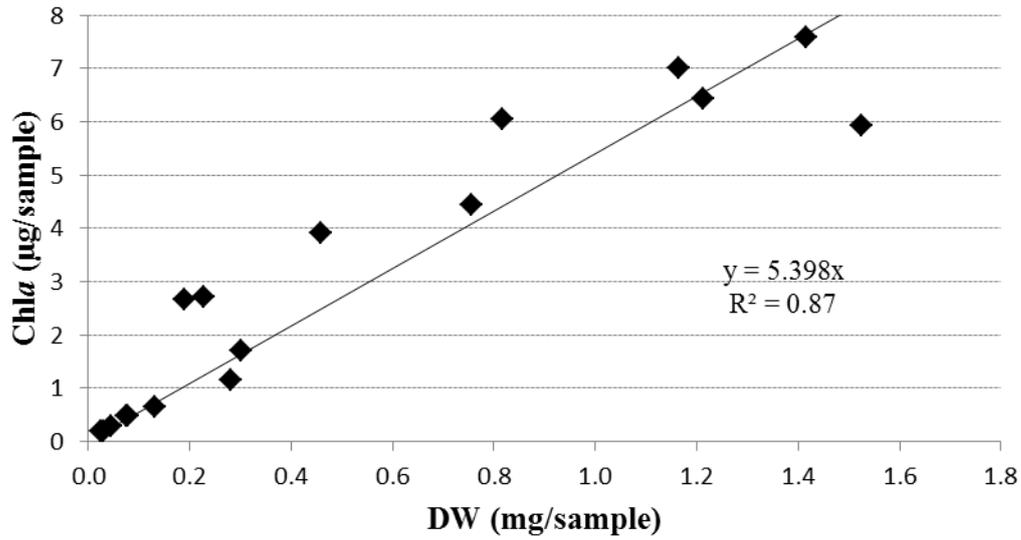
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**b**



511

512 **Fig 2.** Mean ( $\pm$  s.d.) for (a)  $EC_{50}$  and (b)  $EC_{10}$  (mg Cu/L) for periphyton suspension concentrations, ranging from  
513 about 400  $\mu\text{g chl}a/L$  (D4) to about 7000  $\mu\text{g chl}a/L$  (D1) during toxicity tests performed with a 3- and 5-week  
514 periphyton after 2 h of incubation,  $n = 3$ .



515

516 **Fig 3.** Linear relationship between *chl a* (µg/sample) and DW (mg/sample) obtained with 5-week periphyton.  
517 *chl a* concentrations and dry weight are in relation to the total volume of the periphyton suspension used during  
518 toxicity testing.

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