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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

4 Anne Sophie Lambert¹; Stéphane Pesce¹; Arnaud Foulquier^{1, 2}; Josiane Gahou¹; Marina Coquery¹;
5 Aymeric Dabrin¹

6

7 1. Irstea, UR MALY, centre de Lyon-Villeurbanne, 5 rue de la Doua CS 70077, F-69626 Villeurbanne
8 cedex, France

9 2. INRA, UMR Carrtel, 75 av. de Corzent, BP 511. FR-74203 Thonon-les-Bains, France

10

11 **Corresponding Author:**

12

13 Anne-Sophie Lambert: Irstea, UR MALY, 5 rue de la Doua, CS70077, 69626 Villeurbanne Cedex,
14 France; anne-sophie.lambert@irstea.fr; phone +33472208748 ; fax +33478477875

15

16 Other e-mail addresses:

17 stephane.pesce@irstea.fr

18 arnaud.foulquier@irstea.fr

19 josiane.gahou@irstea.fr

20 marina.coquery@irstea.fr

21 aymeric.dabrin@irstea.fr

22

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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 EC₅₀, 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, EC₁₀, and EC₅₀ 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_x 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_x 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_x and periphyton suspension concentration, whatever the exposure
210 duration (Table 1; Fig. 2). For example, after 6 h of incubation, EC₅₀ increased about 14-fold between
211 the most diluted periphyton suspension (D4, mean EC₅₀ = 0.09 mg/L) and the least (D1, mean
212 EC₅₀ = 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 β -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.

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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 μ g/L or DW < 0.4 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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		EC₅₀	EC₁₀
D4	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
D3	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)
D2	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
D1	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.) EC₅₀ and EC₁₀ (mg Cu/L) for periphyton suspension concentrations, ranging from about
 486 400 µg chl_a/L (D4) to about 7000 µg chl_a/L (D1) after 2, 4 and 6 h of incubation. Different letters (a, b, c)
 487 indicate significant difference between EC₁₀ values ($p < 0.05$), $n = 3$, within the D3 dilution.

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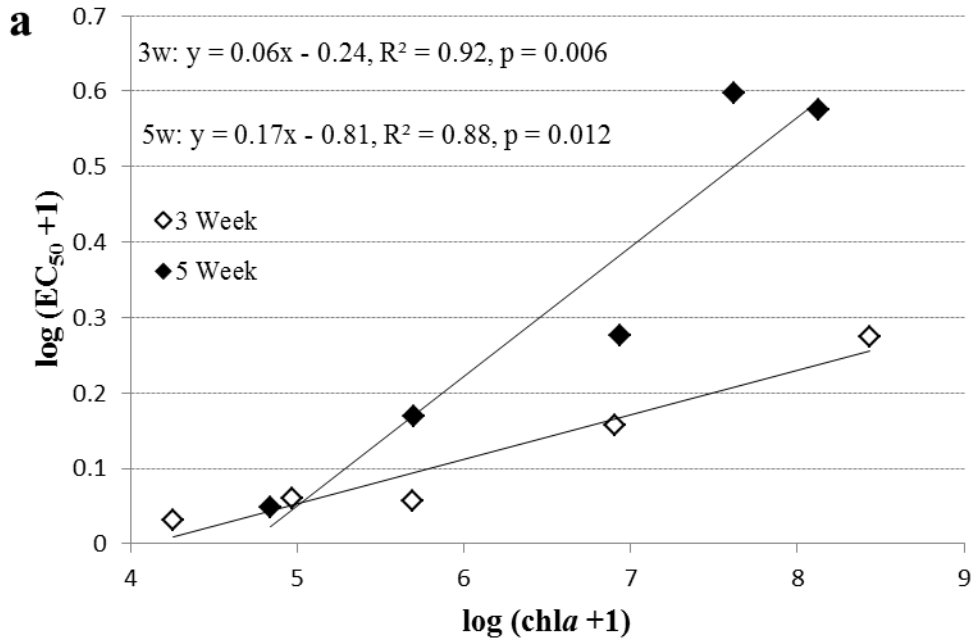
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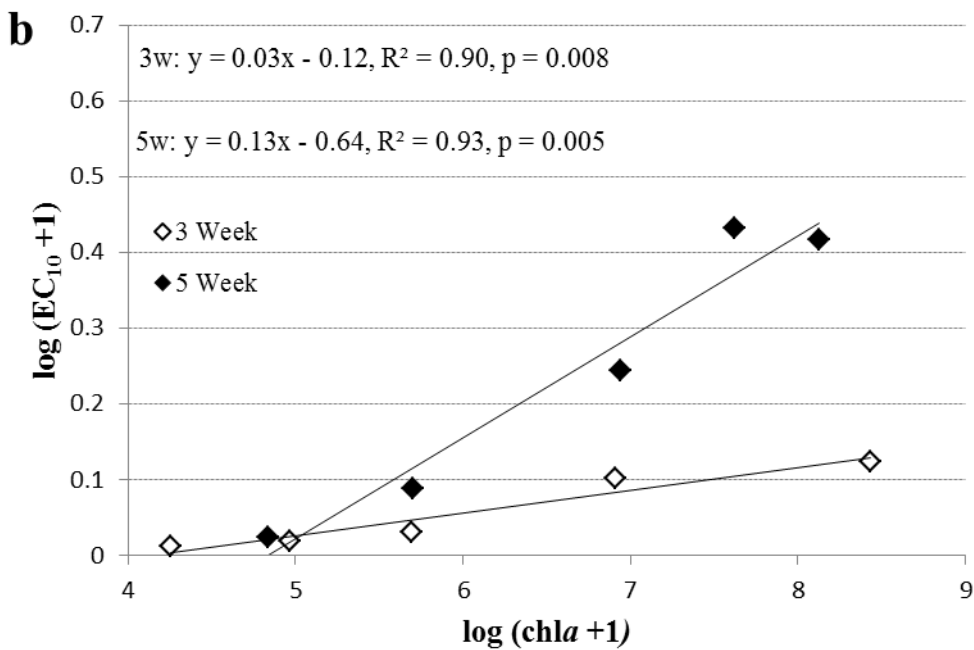
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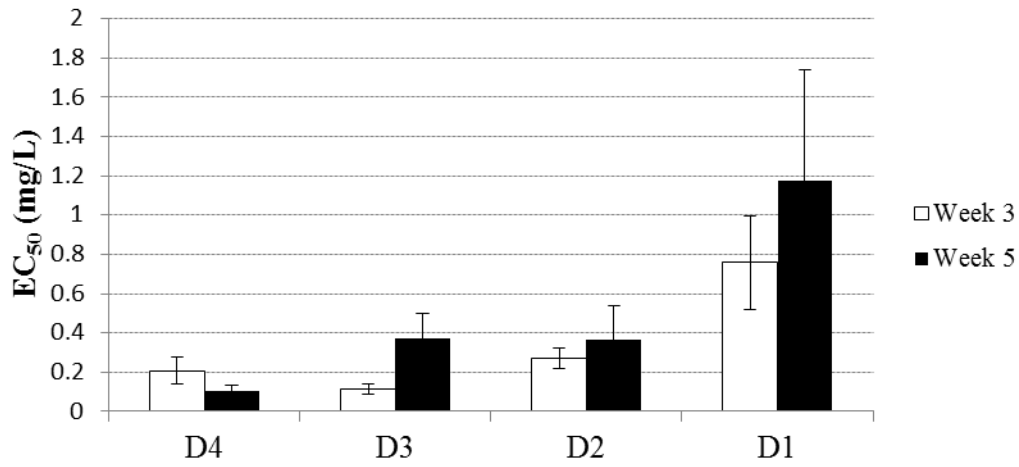


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505 **Fig 1.** Linear relationship between mean *chla* concentrations in periphyton suspension ($\mu\text{g/L}$) and (a) mean EC_{50}
506 and (b) mean EC_{10} (mg Cu/L) during toxicity tests performed with a 3- and 5-week periphyton after 2 h of
507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

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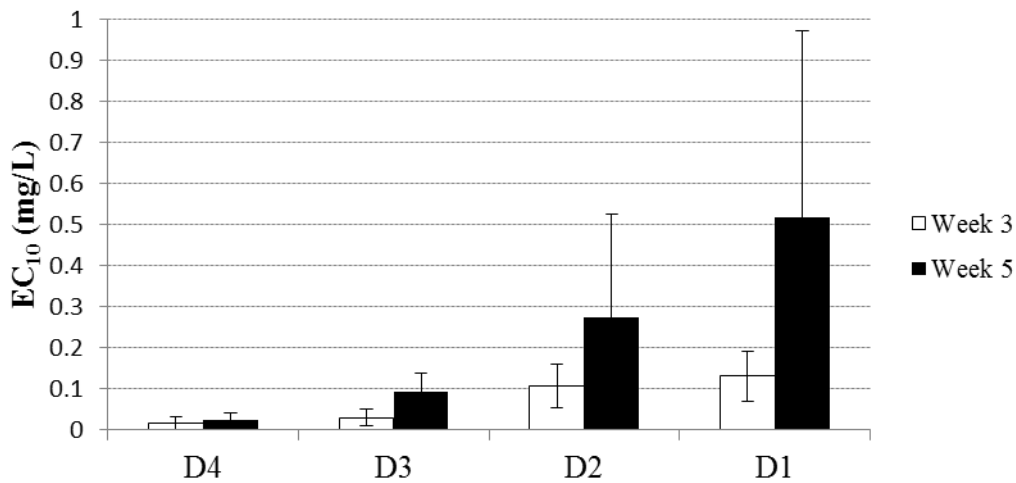
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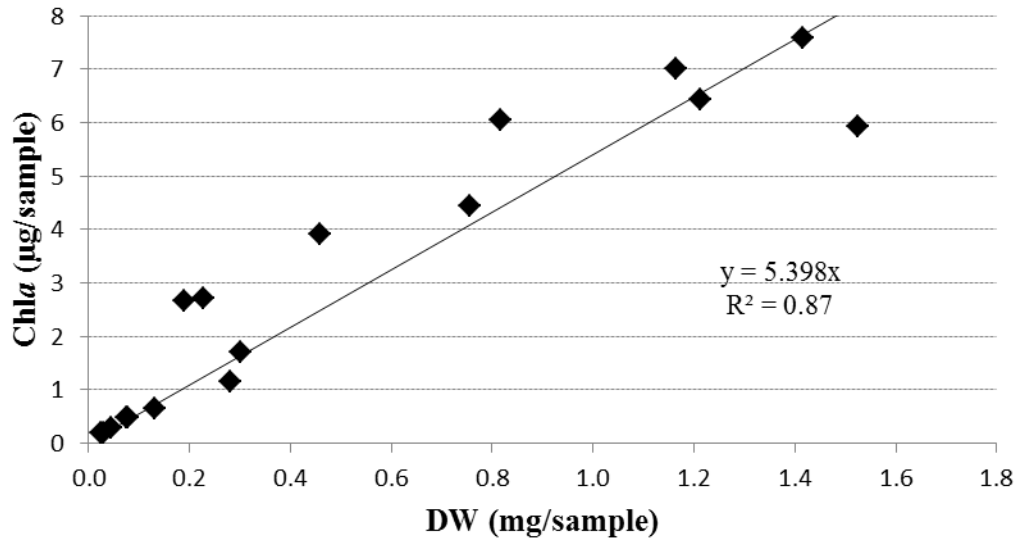
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512 **Fig 2.** Mean (\pm s.d.) for (a) EC_{50} and (b) EC_{10} (mg Cu/L) for periphyton suspension concentrations, ranging from
513 about 400 $\mu\text{g chl}a/L$ (D4) to about 7000 $\mu\text{g chl}a/L$ (D1) during toxicity tests performed with a 3-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



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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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