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

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Abstract

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

Keywords

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

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1 Introduction

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

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

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

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

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Berha 2000; Eklund and Kautsky 2003; Fechner et al. 2010). It is now well accepted that PICT can serve as a powerful tool for *in situ* assessment of chronic effects of toxicants on periphyton communities (Pesce et al. 2010). This kind of approach raises question about the periphyton sampling procedure, which appears to be highly variable between studies, depending on the use of either natural (Dorigo et al. 2010) or artificial substrates, with fluctuating colonization durations (e.g from 2 weeks (Blanck and Dahl 1996) to 2 months (Pesce et al. 2010)). This notably raises the question of the influence of the maturation stage (growth stage) in tolerance assessment. Maturation processes include both species succession, which conditions the tolerance baseline of the community, and the building of a highly complicated structure that can form a protective layer (Extracellular Polymeric Substances), thereby influencing the level of exposure to toxicants (Ivorra et al. 2000; Gold et al. 2003a, 2003b).

Given this background, the main aim of our work was to help identify the most important parameters that must be considered to obtain reproducible and comparable results from short-term toxicity tests for metals on natural phototrophic periphyton communities.

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

2. Materials and Methods

2.1. Experimental setup and periphyton sampling procedure

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

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

2.2. Short-term bioassays

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

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

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

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

The influence of the duration of exposure to Cu was evaluated from toxicity tests after 2 h, 4 h and 6 h of incubation. The influence of the periphyton maturation stage was assessed by performing toxicity 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 $$response = c + \frac{d - c}{1 + \exp\{b \times (\log(Dose) - \log(e))\}}$$

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_{50}, 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_{10}, and EC_{50} 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, EC_{50} increased about 14-fold between
211 the most diluted periphyton suspension (D4, mean EC_{50} = 0.09 mg/L) and the least (D1, mean
212 EC_{50} = 1.23 mg/L), which was characterized by chl*a* concentrations (6980 µg/L) about 17 times

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

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

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

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

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

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3.2. Standardization perspectives

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

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

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results suggest that the relationship between EC_x and chl*a* is probably highly periphyton-dependent. Hence in order to take into account the differences between sampling sites or between sampling periods and thus enable data toxicity comparisons, an *a priori* normalization of the periphyton suspension biomass is advocated.

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

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

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

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

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		EC_{50}	EC_{10}
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

Table 1. Mean (± s.d.) EC_{50} and EC_{10} (mg Cu/L) for periphyton suspension concentrations, ranging from about 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) indicate significant difference between EC_{10} values ($p < 0.05$), $n = 3$, within the D3 dilution.

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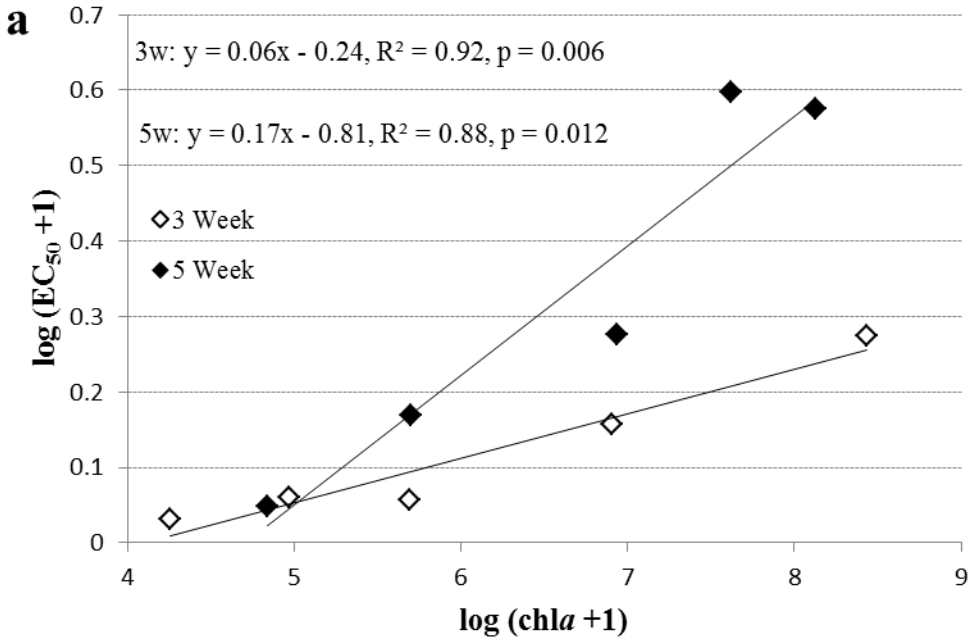

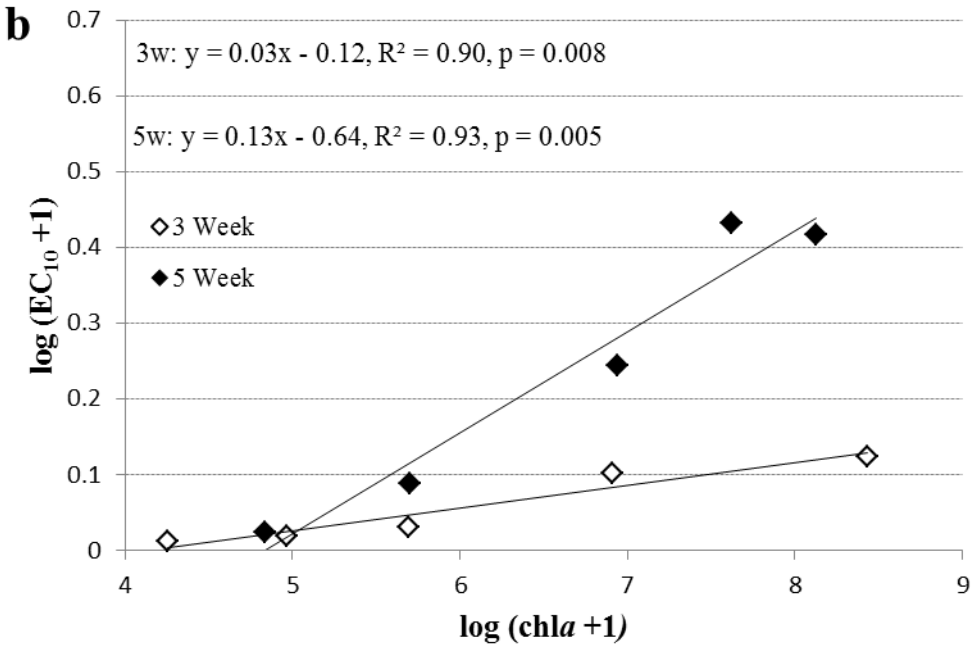

Fig 1. Linear relationship between mean chl*a* concentrations in periphyton suspension (µg/L) and (a) mean EC_{50} and (b) mean EC_{10} (mg Cu/L) during toxicity tests performed with a 3- and 5-week periphyton after 2 h of incubation.

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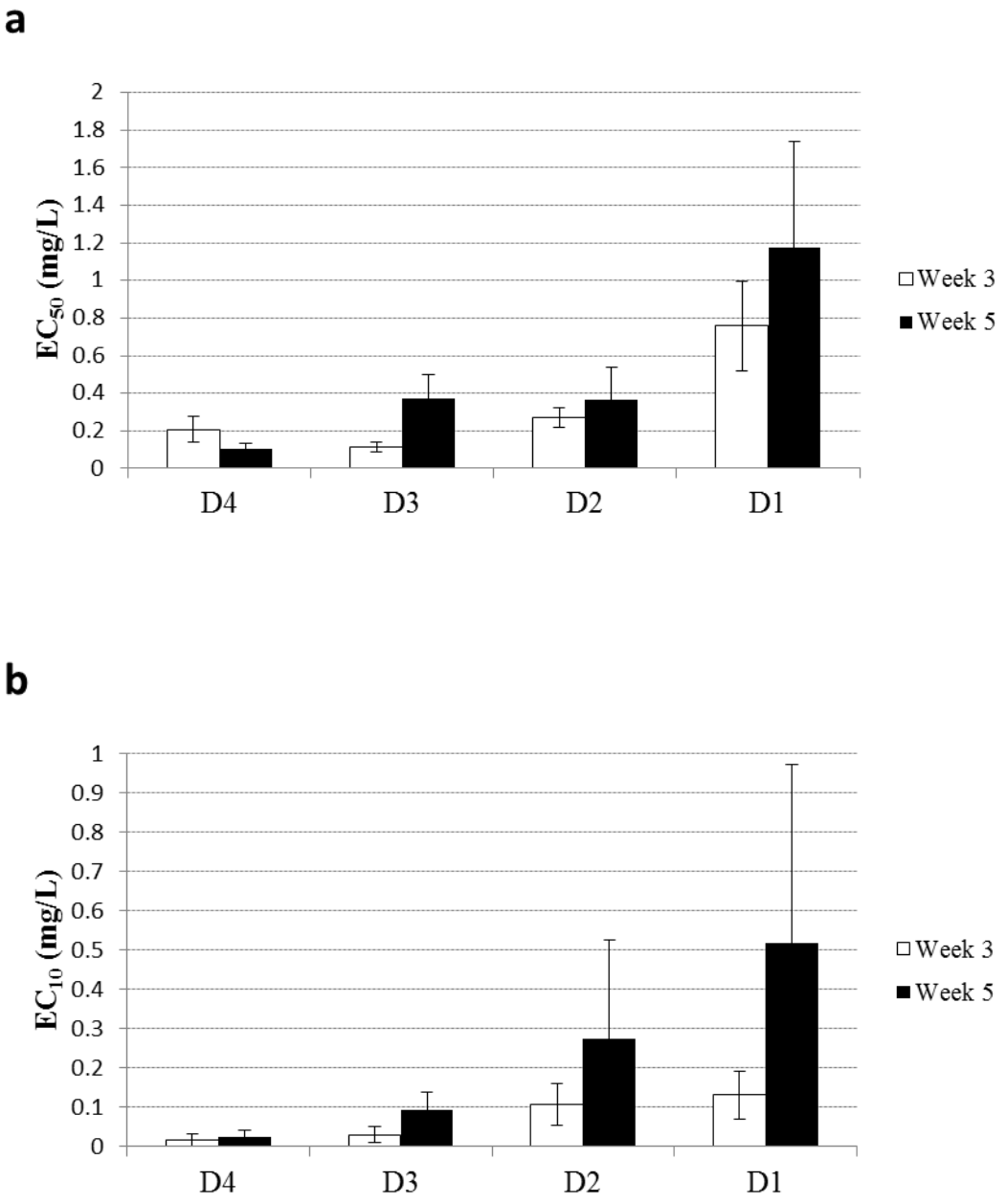

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512 **Fig 2.** Mean (± s.d.) for (a) EC_{50} and (b) EC_{10} (mg Cu/L) for periphyton suspension concentrations, ranging from
513 about 400 µg chl*a*/L (D4) to about 7000 µg chl*a*/L (D1) during toxicity tests performed with a 3- and 5-week
514 periphyton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

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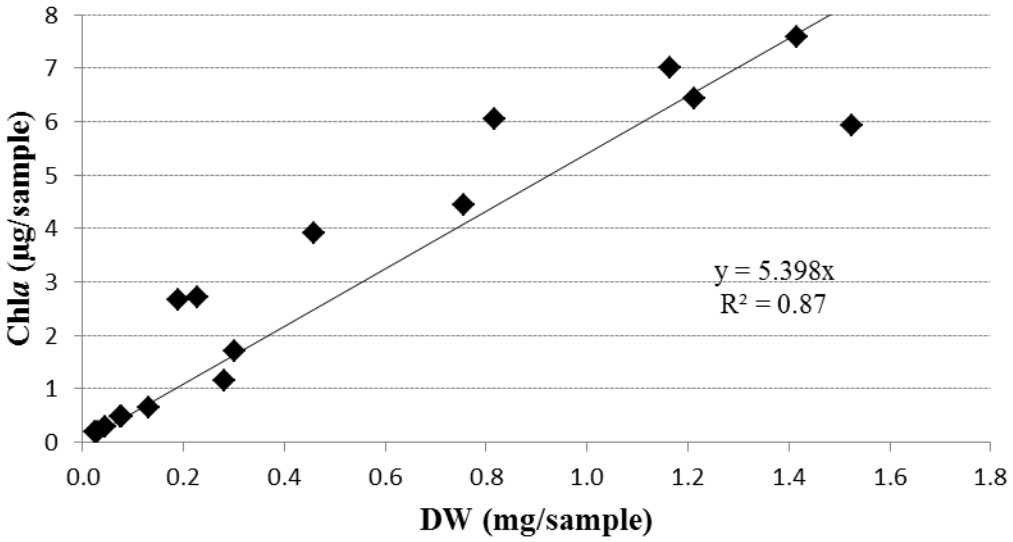

Fig 3. Linear relationship between chl*a* (µg/sample) and DW (mg/sample) obtained with 5-week periphyton. chl*a* concentrations and dry weight are in relation to the total volume of the periphyton suspension used during toxicity testing.