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Pollution-induced community tolerance of freshwater biofilms: Measuring heterotrophic tolerance to Pb using an enzymatic toxicity test

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1 **Pollution-Induced Community Tolerance of freshwater biofilms:**
2 **measuring heterotrophic tolerance to Pb using an enzymatic toxicity test**

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19 **Abstract**

20 This study aims at investigating the impacts of Pb on freshwater biofilms with a Pollution-
21 Induced Community Tolerance (PICT) approach using a recently developed short-term toxicity
22 test based on β -glucosidase activity to measure biofilms' tolerance to Pb. We first investigated
23 more closely the influence of the TSS (Total Suspended Solids) concentrations of the biofilm
24 suspensions used for short-term toxicity tests performed to assess Pb tolerance. The Pb EC₅₀
25 values of four dilutions of the same biofilm suspension increased with their TSS concentrations.
26 TSS-normalization allowed to obtain a unique measure of Pb tolerance, thus confirming that
27 TSS-normalization of EC₅₀ values is a good means to estimate biofilm tolerance to Pb. The
28 experiment was repeated with three different biofilm samples collected at different time and date.
29 Second, biofilms were exposed to Pb (0, 1, 10 and 100 μ g/L) for three weeks in microcosms to
30 assess the impacts of Pb exposure on the communities. An increase in Pb tolerance was observed
31 for the biofilm exposed to 100 μ g/L. Automated Ribosomal Intergenic Spacer Analysis revealed
32 modifications of bacterial and eukaryotic community structure with Pb exposure. Moreover,
33 exposure to 100 μ g/L Pb also led to an increase in Zn tolerance but not Cu tolerance. This study
34 shows that tolerance acquisition to Pb can be detected after exposure to environmental
35 concentrations of Pb using a PICT methodology and normalized EC₅₀ values as measures of Pb
36 tolerance.

37

38 **Key words:** biofilm, metal tolerance, lead, toxicity, PICT, co-tolerance

39 **Introduction**

40 Aquatic systems in urban areas are subjected to chronic and diffuse multi-contamination by
41 mixtures of micropollutants. Those substances are present in river waters at low concentrations
42 which do not always lead to clear biological effects. Being able to assess the impacts of such low
43 contamination levels on aquatic systems is one of the challenges of aquatic ecotoxicology. To
44 address the issue of linking chemical contamination to biological impairment, the Pollution-
45 Induced Community Tolerance (PICT) approach was proposed by Blanck et al. (2002). PICT
46 uses tolerance acquisition at the community level as a biological response to exposure to a toxic
47 agent. An increase in community tolerance to a toxic agent is assumed to be related to the
48 pressure exerted by the toxic agent leading to selection (of tolerant species) or adaptation (both
49 genotypic and phenotypic) processes within the exposed community. Determination of
50 community tolerance is achieved by the means of short-term toxicity tests based on physiological
51 activities (for instance respiration, photosynthesis, etc.) and community tolerance is usually
52 expressed as the EC_{50} value derived from such a short-term test. Comparisons of EC_{50} values
53 obtained from different communities allow to discriminate which community has been exposed
54 to the toxic agent under study or not: an EC_{50} value higher than the EC_{50} value of a control
55 community indicates a PICT effect. The PICT approach has been used on various algal
56 communities exposed to herbicides (for instance Pesce et al., 2010) or on freshwater biofilms, for
57 instance exposed to metals (Fechner et al., 2012a).

58 Biofilms (or periphyton) are complex communities of both autotrophic and heterotrophic
59 microorganisms (algae, bacteria, fungi) that develop on submerged surfaces like rocks,
60 macrophytes or sediments. Biofilms play an important ecological role in aquatic systems both as
61 primary producers (autotrophic microorganisms) and as recyclers of organic matter

62 (heterotrophic microorganisms). They also respond fast to any stress and are thus usually
63 considered as good early-warning indicators of the overall health of aquatic systems (Sabater *et*
64 *al.*, 2002). Biofilms have been used to identify PICT effects with various herbicides, antifouling
65 agents or metals (for a review see Blanck, 2002; Sabater *et al.*, 2007) .

66 In our previous study, a short-term toxicity test based on β -glucosidase activity (β -glucosidase
67 toxicity test) was used to measure metal tolerance levels of heterotrophic communities from
68 freshwater biofilms collected in an urban area (Fechner *et al.*, 2010a) . Biofilms were collected on
69 artificial substrates immersed in the river for several weeks and then scraped to make biofilm
70 suspensions in mineral water. Short-term toxicity tests were performed on those biofilm
71 suspensions. To obtain a reliable measure of community tolerance, a normalized EC_{50} value was
72 proposed in order to take into account modifications of metal bioavailability during the short-
73 term test. Indeed, the issue of bioavailability in the test medium is rarely addressed although
74 modifications of metal bioavailability, for instance related to biofilm composition (Extracellular
75 Polymeric Substances or EPS from the biofilm matrix, organic or mineral particles from the river,
76 etc.), might change any toxicity data derived from the tests. In particular, we observed that EC_{50}
77 values varied with the TSS (Total Suspended Solids) concentration of the biofilm suspensions
78 used for metal toxicity testing. TSS-normalization of EC_{50} values thus appeared as a reliable
79 means to estimate metal tolerance (Fechner *et al.*, 2010a) for metals like Cu, Cd, Ni and Zn. Yet,
80 we concluded that normalization of EC_{50} values might not necessarily be an efficient means to
81 reliably assess Pb tolerance of freshwater biofilms and that further investigations ought to be
82 conducted.

83 Pb has been used for various human activities for centuries and is one of the priority substances
84 of the Water Framework Directive. Several studies suggest that metal bioavailability and toxicity

85 in the presence of natural organic matter is likely to be affected differently in the case of Pb than
86 in the case of other metals like Cu or Zn (for instance Tsiridis et al., 2006; Lamelas et al., 2009;
87 Sánchez-Marín et al., 2010). Indeed, the presence of natural organic matter has been shown to
88 either enhance or decrease Pb toxicity towards aquatic organisms depending on the studies
89 (Sánchez-Marín *et al.*, 2010). Therefore, being able to reliably estimate biofilm tolerance to Pb is
90 a crucial issue in order to assess the impacts of urban metallic exposure on freshwater biofilms.

91 The aim of this study was first to investigate the reliability of Pb tolerance measurements using a
92 PICT approach and the β -glucosidase toxicity test. In order to test the normalization procedure,
93 EC₅₀ values were obtained after short-term exposure to Pb of several dilutions of the same
94 biofilm suspension and the experiment was repeated with three different biofilm samples. EC₅₀
95 values were expected to increase with TSS concentrations of biofilm suspensions used for metal
96 toxicity tests thus confirming that TSS-normalized EC₅₀ values provide a reliable measure of
97 community tolerance. Second, we wanted to confirm those results by investigating the impacts of
98 Pb exposure in terms of modifications of Pb tolerance after a three-week exposure to Pb in a
99 microcosm experiment. Modifications of both Zn and Cu tolerance and of bacterial and algal
100 community structures (using Automated Ribosomal Intergenic Spacer Analysis or ARISA) were
101 also investigated.

102

103 **Materials and Methods**

104

105 ***Experiment design***

106 Two separate experiments were performed on freshwater biofilms. In experiment 1, natural
107 freshwater biofilms were collected *in situ* and we investigated the influence of biofilm

108 suspensions' concentrations on Pb tolerance measurements using the β -glucosidase toxicity test.
109 In experiment 2, periphyton was grown and exposed to Pb under controlled conditions to assess
110 the impacts of Pb exposure in terms of tolerance acquisition and modifications of community
111 structure.

112

113 ***Experiment 1: TSS normalization of Pb EC₅₀ values***

114 *Biofilm sampling*

115 Natural freshwater biofilms were collected on three sampling sites located in the vicinity of Paris
116 (North of France): site 1 (Saint-Maurice) is located in the Marne river upstream from Paris
117 (Strahler: 6, mean annual flow: 110 m³/s), site 2 (Bougival) is located in the Seine river
118 downstream from Paris (Strahler: 7, mean annual flow 180 m³/s) and site 3 (Saint-Cyr-sous-
119 Dourdan) is located in the Rémarde river (Strahler: 3, mean annual flow: 0.6 m³/s). Those three
120 sites were chosen to provide three different environments for biofilm growth. Metallic
121 concentrations were not monitored during experiment 1, yet recent contamination data from those
122 3 sites are provided elsewhere (Fechner et al., 2011; Fechner et al., 2012a; Fechner et al., 2012b)
123 showing that site 2 is more contaminated than sites 1 and 3.

124 Biofilms were grown on Low Density PolyEthylene (LDPE) membranes (30×10 cm²) vertically
125 attached to plastic crates immersed in the river at the three sampling sites as in Fechner et al.
126 (2010a). Biofilms were collected at each site on different dates after several weeks of *in situ*
127 colonization (> 4 weeks). 3 to 6 colonized membranes were collected at each site and carried
128 back to the laboratory in 250 mL glass bottles filled with mineral water (Grand Barbier,
129 Montdore, France). They were then carefully hand-scraped to remove periphyton. Biofilm
130 suspensions in mineral water were magnetically homogenized and aliquoted for further analyses.

131

132 *Biofilm parameters*

133 At each sampling date, biofilm dry weights (DWs) were measured in triplicate as in Fechner et al.
134 (2010b). Biofilm suspensions used for the toxicity tests were characterized by their TSS

135 concentrations expressed in gram of dry matter per liter of biofilm suspension. TSS
136 concentrations were calculated simultaneously with DW concentrations by filtration of a known
137 volume of suspension.

138

139 *Dilution experiments: influence of the periphyton suspension concentration*

140 For each of the three biofilm samples, four dilutions were prepared in mineral water from a
141 unique biofilm suspension and used to perform four β -glucosidase toxicity tests and determine
142 four EC₅₀ values (see below).

143

144 *Pb tolerance measurements using the β -glucosidase toxicity test*

145 Pb EC₅₀ values of biofilm samples were obtained using the results from the β -glucosidase toxicity
146 test as in Fechner et al. (2010a). Briefly, biofilms were exposed for one hour to acute
147 concentrations of Pb (six concentrations of metal varying between 0.001 and 10 mM were tested
148 in triplicate for each toxicity test). Pb exposure levels during the toxicity tests were checked by
149 measuring metal concentrations in the stock solutions by flame AAS (Varian Inc., USA). After
150 one hour of exposure, β -glucosidase activity of the metal-exposed biofilms was measured
151 spectrofluorometrically using Methylumbelliferyl- β -D-glucopyranoside (MUF-GLU) (Sigma-
152 Aldrich). Fluorescence of 4-Methylumbelliferone (MUF) was measured using an LB 941 Tristar
153 Ti fluorescence microplate reader (Berthold Technologies, Bad Wildbad, Germany)
154 (excitation/emission filters: 355 and 460 nm). Fluorescence measurements were converted into
155 MUF concentrations by calibrating the spectrofluorimeter with a range of MUF solutions
156 prepared in demineralised water. % inhibition of β -glucosidase activity was then calculated using

157 MUF concentrations measured for each metal concentration tested. 50% effect concentrations
158 (EC₅₀) and maximum inhibition levels were determined from the dose/effect curves, which were
159 obtained by plotting the % inhibition of β-glucosidase activity as a function of metal
160 concentration, and by fitting the data to the Hill model as described elsewhere (Fechner *et al.*,
161 2010a). The general form of the curve fitted on the data is:

$$162 \quad F(x) = \% \max \times \frac{x^H}{x^H + EC_{50}^H}$$

163 where x is the metal concentration, %max is the predicted effect when the toxicant concentration
164 is infinite, and H is the Hill number (slope of the toxicity curve). Each parameter (%max, EC₅₀
165 and H) was adjusted for the calculation by non-linear regression. 95% confidence intervals
166 around the fitted parameters were estimated using a Bootstrap method. Parameters (EC₅₀ values
167 and maximum % inhibition) were considered significantly different when 95% confidence
168 intervals were not overlapping.

169 TSS-normalized EC₅₀ values were calculated by dividing each EC₅₀ value by the TSS
170 concentration of the biofilm suspension used for Pb toxicity testing. Data from the four toxicity
171 tests were also gathered on the same plot to produce a unique dose/effect curve with the %
172 inhibition of β-glucosidase activity as a function of TSS-normalized metal concentrations from
173 the four toxicity tests. A unique normalized EC₅₀ value was also calculated from this dose/effect
174 curve for each biofilm sample.

175

176 ***Experiment 2: long-term exposure to Pb in microcosms***

177 *Biofilm exposure to Pb in microcosms*

178 A biofilm suspension obtained from site 3 was used to inoculate microcosms (10L plastic tanks)
179 at the beginning of experiment 2. Site 3 was chosen because it is relatively unaffected by the
180 urban area of Paris in terms of metallic contamination (Fechner *et al.*, 2011).

181 The medium used to cultivate biofilm in the microcosms was mineral water (Aquarel, Nestlé
182 Waters, France) enriched with nutrients (N, P, Si) so as to reach concentrations of 1 mg/L PO_4^{3-} ,
183 20 mg/L NO_3^- and 32 mg/L SiO_2 , which were close to nutrient concentrations measured at site 3/
184 Aquarel mineral water was chosen for the medium so that physico-chemical parameters were
185 roughly similar to those from the river at site 3 (Fechner *et al.*, 2011).

186 In each microcosm, periphyton grew on clean immersed LDPE membranes (140 cm², 14
187 membranes per aquarium) at 20°C, under continuous water mixing conditions using water pumps
188 (Eheim Compact 300, 300 L/h) and a light regime of 13h/11h light/dark cycle using neon lamps
189 (Sylvania, Gro-Lux, F14W-GRO-T8) during three weeks. The medium was changed weekly
190 during the three weeks of colonization. Prior to the beginning of experiment 2, indoor plastic
191 aquaria and LDPE membranes were rinsed with 10% HNO_3 , then twice with distilled water, and
192 finally left to equilibrate for 24h with the cultivation medium spiked with Pb to limit adsorption
193 of the metal on the containers' walls.

194 Cultivation medium was spiked with stock solutions of PbNO_3 to expose biofilm to three Pb
195 concentrations (1, 10 and 100 µg Pb/L nominal concentrations). One replicate microcosm was
196 used per treatment and a fourth microcosm was used as control (aquaria filled with metal-free
197 medium), with one replicate microcosm per treatment. Pb concentrations in the aquaria were
198 measured daily in aliquots of 5 mL of water: the decrease in metal concentration, mainly due to

199 metal absorption by biofilm, was corrected by adding stock solutions of PbNO₃ as required
200 (≤100μL per aquarium).

201 Exposure concentrations were chosen so as to remain realistic from an environmental point of
202 view for the study of aquatic systems under diffuse urban pressure such as the Seine river in the
203 area of Paris, where total Pb concentrations reaching 5 μg/L have been recorded (Fechner et al.,
204 2012a).

205

206 *Laboratory analyses*

207 Water samples were collected daily from the aquaria for measurement of physico-chemical
208 parameters (major anions and cations) and metal concentrations. Pb concentrations were
209 measured daily on 5-mL samples acidified to 1% HNO₃ (suprapure, Merck) by graphite furnace
210 Atomic Absorption Spectrometry (AAS). Validity of the method was checked daily with a
211 certified reference material (EP-L-2, SCP Science).

212

213 *Biofilm parameters*

214 After three-weeks of biofilm growth, the colonized membranes from each microcosm were
215 scraped in a known volume of mineral water (Grand Barbier, Montdore, France) as in Fechner et
216 al. (2010a) to form a single biofilm sample per aquarium which was homogenized by magnetic
217 stirring. Biofilm samples are hereafter referred to as Pb 0 (control), Pb 1, Pb 10 and Pb 100
218 according to Pb exposure levels. Aliquots of biofilm suspensions were assigned to various
219 analyses in terms of biomass (DW and Ash-Free Dry Weight or AFDW), metal accumulation and
220 tolerance acquisition were determined as in Fechner et al. (2011). To perform ARISA, three

221 aliquots of 50 mL of each biofilm suspension were centrifuged for 15 minutes at 10 000 g, and
222 4°C. Supernatants were discarded and pellets were frozen and stored at -80°C for further use.

223 AFDW and Pb accumulation in biofilms were performed as in Fechner et al. (2011). Intracellular
224 or non-exchangeable metal concentrations were estimated after washing biofilm samples with
225 EDTA at 4 mM for 10 min according to Meylan et al. (2004).

226

227 *Tolerance measurements with β -glucosidase toxicity test*

228 Pb tolerance levels of Pb 0, Pb 1, Pb 10 and Pb 100 biofilms were assessed by the means of the β -
229 glucosidase toxicity test as in experiment 1. Community tolerance to metals was assessed by
230 calculation of normalized EC₅₀ values (by dividing each EC₅₀ value by the TSS concentration of
231 the periphyton suspension used for the short-term test). Cu and Zn tolerance levels of both Pb 0
232 and Pb 100 biofilms were also measured using the β -glucosidase toxicity test and by calculating
233 TSS-normalized Cu and Zn EC₅₀ values.

234

235 *ARISA fingerprinting of periphyton*

236 DNA extraction, PCR-amplification and ARISA was performed on triplicates of biofilm pellets
237 as in Fechner et al. (2011). Briefly, primers ITSF/ITSReub (Cardinale *et al.*, 2004) were used for
238 amplification of bacterial 16S-23S intergenic spacers and primers 2234C/3126T (Ranjard *et al.*,
239 2001) were used for amplification of eukaryotic ITS1-5.8S-ITS2 regions. PCR-amplified
240 fragments were separated on an electrophoresis Bioanalyzer (2100 Electrophoresis Bioanalyzer,
241 Agilent Technologies, Santa Clara, CA) and fluorescence data was converted into
242 electrophoregrams using 2100 Expert software (Agilent Technologies, Santa Clara, CA, US).

243 Comparisons between ARISA fingerprints (either bacterial or eukaryotic) were made using the
244 Jaccard index as a % of similarity between two fingerprints from the same experiment using the
245 2100 Expert software (Agilent Technologies, Santa Clara, CA, US): $D(i,j) = a/(a+b+c)$, where a =
246 number of fragments contained in both samples, b = number of fragments contained in sample i
247 but not j, c = number of fragments contained in sample j but not i. This allows comparison
248 between fingerprints using solely the presence or absence of fluorescence peaks and without
249 taking into account peak intensity. To include the maximum number of peaks while excluding
250 background fluorescence, only peaks with a fluorescence value superior to 20 FU (Fluorescence
251 Units) were considered for calculation of the Jaccard indexes. ARISA fragment lengths differing
252 by more than 5 bp were considered distinct.

253 Electrophoregrams in ASCII formats were also imported into the StatFingerprints R package
254 (Michelland *et al.*, 2009) using the 2.10.1 version of the R project. ARISA profiles were aligned
255 and the area under each curve was normalized to 1 before calculation of Shannon indexes, to

256 assess community diversity using the area under peaks. For calculation of Shannon indexes, all
257 fluorescence peaks were considered (no fluorescence threshold).

258

259 **Results and discussion**

260 *Experiment 1: normalization of Pb tolerance measurements on freshwater biofilms*

261 Our previous study (Fechner et al., 2010a) showed that EC₅₀ values obtained on a given biofilm
262 sample vary with the concentration of the biofilm suspension used for metal toxicity testing. Yet,
263 using the PICT approach requires reliable comparisons of tolerance levels. It is therefore
264 important to develop a measure of community tolerance that does not depend on experimental
265 parameters like the concentration of the suspension used for metal toxicity testing. Our results
266 show that, for Pb also, the higher the TSS concentration of the biofilm suspension, the higher the
267 EC₅₀ value obtained (Figure 1 and Table 1). As observed previously with Cd, Cu, Ni and Zn,
268 mineral or organic particles from the biofilm suspension seem to affect Pb bioavailability during
269 the short-term test. Therefore, when the suspension has a high TSS concentration, Pb
270 bioavailability is smaller and the EC₅₀ value obtained is higher. TSS concentration provides a
271 rough estimate of mineral or organic matter that modifies metal bioavailability during short-term
272 metallic exposure without presuming on the nature of the matter considered, which could be
273 either organic or mineral particles from the river, EPS from the biofilm matrix, etc. This tendency
274 was observed with three different biofilm samples which validates the fact that the influence of
275 the biofilm suspension's concentration on Pb tolerance measurement is not related to the
276 particular composition of a biofilm sample but to modifications of metal bioavailability during
277 the toxicity test.

278 Defining a TSS-normalized EC_{50} value as a measure of biofilm tolerance therefore appears as a
279 reliable means to reduce the variability of tolerance measurements due to experimental factors.
280 Indeed, the TSS concentration of the biofilm suspension used for metal toxicity testing is related
281 to the number and size of colonized membranes that were collected to sample periphyton, the
282 volume of mineral water used to prepare the suspension, and mostly biofilm growth in the river,
283 which cannot be controlled. Figure 1 shows how TSS-normalization enables to merge data from
284 four toxicity tests performed on four dilutions of the same biofilm sample (left graphs) into a
285 unique dose/effect curve (right graphs) simply by representing the inhibition percentage of β -
286 glucosidase activity as a function of TSS-normalized Pb concentrations (right graphs) instead of
287 Pb exposure concentrations (left graphs).

288 With TSS-normalization, the variability of Pb tolerance measurements with the concentration of
289 the biofilm suspension used for metal toxicity testing is considerably reduced (Table 1). Results
290 from experiment 1 confirm that Pb normalized EC_{50} value are robust and reliable measures of
291 river biofilm tolerance to Pb.

292

293 ***Experiment 2: impact of Pb exposure on biofilm tolerance and community structure***

294 Experiment 2 aimed at assessing the impacts of Pb on freshwater biofilm after a long-term
295 exposure in microcosms using the β -glucosidase toxicity test. Pb concentrations measured in the
296 exposed biofilms confirm that they accumulated Pb during exposure (Table 2). Yet Pb
297 accumulation was much smaller than previously observed for metals like Cd, Ni and Zn (Fechner
298 et al., 2011). For instance, the bioconcentration factor (total biofilm-accumulated metal in $\mu\text{g/g}$
299 DW divided by the nominal metal concentration in the corresponding microcosm in $\mu\text{g/L}$) varied

300 from 0.50 (Pb 1) to 0.06 (Pb 10 and Pb100) whereas in our previous study the bioconcentration
301 factor reached 11 and 40 for respectively Cd and Zn-exposed biofilms (Fechner et al., 2011).
302 Moreover, Pb accumulation was not significant for biofilms exposed to 1 and 10 µg/L Pb (Table
303 2).

304 Pb exposure was accompanied by modifications of community structures as revealed by ARISA
305 for both bacterial and eukaryotic communities. Indeed both the Shannon index and the number of
306 fluorescence peaks tended to decrease for Pb-exposed biofilms especially for the Pb 100 biofilm
307 (Table 3). Moreover, bacterial community structure was influenced by metal exposure as Pb 0
308 (control) and Pb 100 (highest exposure level) biofilms had the lowest percentage of similarity
309 (Jaccard index, Table 4) whereas Pb 10 and Pb 100, which correspond to the two highest
310 exposure levels, shared the highest percentage of similarity. As far as eukaryotic communities are
311 concerned, the percentage of similarity was surprisingly high for Pb 0 and Pb100 biofilms but
312 was also high for Pb 10 and Pb 100 biofilms. Exposure to Pb also induced a decrease of β-
313 glucosidase activity especially for the Pb 100 biofilm (Table 2) which indicates a functional
314 impact of Pb exposure at 100 µg/L Pb. DW and AFDW also decreased for the Pb 100 biofilm but
315 increased slightly for Pb 1 and Pb 10 communities. A similar effect was observed on DW for
316 biofilms exposed to low metal concentrations (Morin et al., 2008; Fechner et al., 2011).

317 The β-glucosidase toxicity test proved to be an efficient tool to detect Pb-induced tolerance. Two
318 biological responses can be derived from the toxicity test: a measure of community tolerance
319 (normalized EC₅₀ value) and a maximum % inhibition of β-glucosidase activity (Table 5). A
320 maximum % inhibition inferior to 100% means that β-glucosidase activity cannot be fully
321 inhibited by short-term exposure to metals even at high exposure concentrations (the highest
322 metal concentrations tested are 10 mM namely 2.07 g/L for Pb). This phenomenon was already

323 observed in previous studies on laboratory or *in situ* exposed biofilms with the same toxicity test
324 (Fechner et al., 2011; Fechner et al., 2012a). In the present study, β -glucosidase toxicity tests
325 revealed the impact of Pb exposure on the Pb 100 biofilm with a 1.7 increase of normalized EC_{50}
326 value and a significant decrease of maximum % inhibition (72.30 %) when compared to Pb 0
327 (Figure 2 and Table 5).

328 The maximum inhibition % remained close to 100 % for Pb 0, Pb 1 and Pb 10 biofilms and no
329 increase of the normalized EC_{50} value was observed after exposure to 1 and 10 $\mu\text{g/L}$ Pb (Table
330 5). A similar phenomenon was observed for Cd, Ni and Zn in our previous study: tolerance
331 acquisition to each metal could only be observed for high exposure levels: 3 $\mu\text{g/L}$ Cd, 50 $\mu\text{g/L}$
332 Ni, 200 $\mu\text{g/L}$ Zn but not for low exposure levels (0.3, 5 and 20 $\mu\text{g/L}$ respectively) (Fechner et al.,
333 2011). Yet those metallic exposure levels remain environmentally-realistic and much smaller
334 than those usually tested in the scientific literature as discussed previously (Fechner et al., 2011).
335 Moreover, those results do not question the sensitivity of tolerance measurement to assess the
336 effects of lower metallic exposures. Indeed, metal tolerance acquisition was already observed in
337 the field at diffuse concentration levels using the same methodology (Fechner et al., 2012a).
338 Therefore, experiment 2 validates the use of the β -glucosidase toxicity test to assess Pb tolerance
339 acquisition at environmental metal concentrations.

340 Cu and Zn tolerance levels were measured on Pb 0 and Pb 100 biofilms to investigate co-
341 tolerance (Table 5 and Figure 3). The Pb 100 biofilm became tolerant to Zn (3.3 increase of the
342 normalized EC_{50} value when compared to the Pb 0 biofilm and a decrease of the maximum %
343 inhibition to 88.3 %). As far as Cu is concerned, no modification of the normalized EC_{50} value
344 was observed but the % maximum inhibition decreased to 78.3 after short-term exposure to Cu.
345 Those results indicate Pb-induced modifications in the response of the heterotrophic community

346 to Cu. Co-tolerance is a common phenomenon for algal and bacterial microorganisms and has
347 been previously observed on biofilms (for instance Soldo and Behra, 2000; Fechner et al., 2011) .
348 It can be related to various defense mechanisms either specific (for instance metal efflux or
349 decrease of metal uptake by modification of channel or carrier proteins used by the metal ions to
350 enter the cells (Harrison et al., 2007) or non-specific, for instance production of metal-binding
351 EPS (Mason and Jenkins, 1995; Jang et al., 2001) to a metal. The fact that exposure to Pb
352 induced tolerance to both Pb and Zn but not to Cu suggests that, in this case, the exposed
353 community probably developed defense mechanisms that are specific to Pb and Zn. Some of the
354 mechanisms that are described in the literature might explain co-tolerance to both Pb and Zn: Zn-
355 CPx-type ATPases which transport Zn^{2+} , Cd^{2+} and Pb^{2+} from the cytoplasm to the outside of the
356 cell or to the periplasm could be involved (Rensing et al., 1999; Nies, 2003). For instance, PbrA,
357 one of the Zn-CPx-type ATPases, was shown to be part of a plasmid-mediated Pb resistance
358 determinant in *R. metallidurans* (Nies, 2003). Co-tolerance to Zn had previously been described
359 using the same methodology for tolerance measurements after exposure to Cd (Fechner et al.,
360 2011). Co-tolerance has implications for use of tolerance measurements to assess the impacts of
361 toxic exposure in the field. Indeed, it means that proper identification of the exact selection
362 pressure is difficult. In the present study, tolerance to Zn is induced by exposure to Pb and not by
363 exposure to Zn. In field studies in a context of urban multi-contamination, tolerance to Zn can
364 therefore not be directly linked to Zn exposure levels. Co-tolerance mechanisms are numerous
365 and multiple patterns of co-tolerance might be observed depending on exposure concentrations
366 and biofilm composition. It is thus probable that metal tolerance has to be considered as an early-
367 warning response to a mixture of metals but cannot be used to investigate exposure to a specific
368 metal.

369

370 **Conclusion**

371 This study focused on the assessment of robust Pb tolerance measurements on freshwater
372 biofilms. Our results show that the methodology previously developed (tolerance measurement
373 with the β -glucosidase toxicity test and TSS-normalization of EC_{50} value) can be used to measure
374 Pb tolerance. Experiment 1 confirms that TSS-normalized EC_{50} values can be reliably used to
375 compare Pb tolerance levels of different biofilm samples. Moreover, the methodology allowed
376 the detection of tolerance acquisition after long-term exposure to Pb under controlled conditions
377 (experiment 2). Co-tolerance to Zn was observed for the biofilm exposed to 100 $\mu\text{g/L}$ Pb, but no
378 modification of Cu tolerance was observed. Tolerance acquisition was accompanied by
379 modifications of community structure as revealed by fingerprinting and by the appearance of
380 maximum % inhibitions of β -glucosidase activity inferior to 100%. This parameter appears as an
381 interesting biological response obtained with the β -glucosidase toxicity test. It was indeed
382 observed after short-term exposure to Cu for the biofilm exposed to 100 $\mu\text{g/L}$, whereas no
383 increase of the EC_{50} value was detected. This study shows that the impacts of Pb exposure can be
384 detected after exposure to environmental concentrations of Pb using a PICT approach.

385

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390 **Table 1.** EC₅₀ and normalized EC₅₀ (with their 95% confidence intervals) obtained during
 391 experiment 1 on biofilms collected at sites 1, 2 and 3. EC₅₀ values are expressed in mgPb/L and
 392 normalized EC₅₀ values in en mgPb/gTSS.

393

Sampling site	TSS concentration (standard deviation) of each biofilm suspension	EC ₅₀ (and 95% confidence intervals)	Normalized EC ₅₀ (and 95% confidence intervals)	Global normalized EC ₅₀ from the unique dose/effect curve
Site 1 (Marne river)	17.63 (0.14)	525.01 (434.52 - 651.51)	29.79 (22.59 - 36.98)	39.32 (34.41 - 46.98)
	5.98 (1.08)	177.64 (159.59 - 201.23)	29.70 (18.48 - 40.93)	
	2.78 (0.41)	116.16 (101.38 - 140.43)	41.76 (28.55 - 54.98)	
	1.03 (0.10)	70.98 (53.41 - 86.80)	68.91 (47.76 - 90.06)	
Site 2 (Seine river)	13.94 (0.49)	329.00 (192.34 - 456.88)	23.60 (13.66 - 33.54)	33.54 (28.73 - 38.76)
	2.84 (0.06)	70.86 (62.43 - 79.35)	24.98 (21.79 - 28.17)	
	1.24 (0.10)	47.72 (38.53 - 57.74)	38.55 (28.41 - 48.69)	
	0.49 (0.05)	21.59 (17.44 - 26.11)	43.61 (30.53 - 56.69)	
Site 3 (Rémarde river)	1.75 (0.17)	149.92 (136.53 - 166.88)	85.48 (66.91 - 104.05)	85.36 (79.45 - 91.87)
	1.27 (0.45)	100.26 (93.43 - 106.49)	79.26 (24.29 - 134.23)	
	0.90 (0.01)	59.67 (47.15 - 86.42)	66.06 (36.38 - 95.74)	
	0.34 (0.07)	34.25 (28.53 - 48.58)	99.75 (42.42 - 157.08)	

394

395 **Table 2.** Impact of Pb on biofilm parameters: DW, AFDW (both expressed in μg dry matter/ cm^2
 396 of colonized membrane) and β -glucosidase activity (expressed in μM MUF/g of organic matter/h)
 397 and on biofilm-accumulated Pb concentrations (total and intracellular) expressed in $\mu\text{gPb/g}$ of dry
 398 matter (DW). Statistical differences were assessed using a non-parametric test (Kruskal-Wallis
 399 followed by the Dunn test for pairwise comparisons of means) as few replicates were measured
 400 ($n= 3$). Different letters indicate significant differences between biofilm samples ($p = 0.05$). No
 401 statistical differences was be assessed for biofilm-accumulated metals as $n = 2$.

402

metal treatment	biofilm parameters			biofilm-accumulated metals	
	DW	AFDW	β -glucosidase activity	total	intracellular
Pb 0	179.37 ^{ab} (22.50)	75.40 ^{ab} (0.75)	169.60 ^a (0.73)	0.56	0.70
Pb1	287.30 ^{ab} (24.09)	95.24 ^{ab} (0.95)	92.98 ^{ab} (5.50)	0.50	0.52
Pb 10	461.31 ^a (16.57)	150.99 ^a (1.51)	151.36 ^{ab} (2.83)	0.59	0.49
Pb 100	150.79 ^b (10.41)	44.97 ^b (0.45)	20.18 ^b (0.30)	6.47	4.26

403

404

405

406 **Table 3.** Shannon-Weaver index and number of major fluorescence peaks (fluorescence > 20
407 FU) from ARISA profiles of Pb 0, 1, 10 and 100 biofilms from experiment 2. Standard deviations
408 are shown in brackets (n = 3).

409

Pb exposure levels	Bacteria		Eukaryotes	
	Shannon index	Number of peaks	Shannon index	Number of peaks
Pb 0	2.14 (0.15)	8	1.78 (0.14)	9
Pb 1	2.14 (0.34)	8	1.19 (0.004)	7
Pb 10	2.02 (0.4)	5	1.55 (0.05)	7
Pb 100	1.5 (0.03)	4	1.34 (0.06)	8

410

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412

413 **Table 4.** % of similarity between (bacterial and eukaryotic) ARISA profiles corresponding to the
414 four exposure concentrations (experiment 2).

415

% of similarity	Bacteria	Eukaryotes
Pb 0/Pb 1	28.57%	14.29%
Pb 0/Pb 10	27.27%	40.00%
Pb 0/Pb 100	18.18%	45.45%
Pb 1/Pb 10	33.33%	18.18%
Pb 1/Pb 100	25.00%	16.67%
Pb 10/Pb 100	37.50%	44.44%

416 **Table 5.** Normalized EC₅₀ values and maximum inhibition levels of β-glucosidase activity
 417 calculated from the dose/effect curves. with their 95% confidence intervals (experiment 2).

Pb exposure levels	Short-term toxicity tests	normalized EC ₅₀ in mg _{metal} /g _{TSS} (95% confidence interval)	% maximum inhibition level (95% confidence interval)
Pb 0	Pb	15.81 (13.18-18.44)	98.92 (98.01-99.78)
Pb 1		17.73 (7.61-27.86)	92.38 (90.85-93.65)
Pb 10		15.76 (13.62-17.90)	99.01 (97.71-100)
Pb 100		26.64 (16.40-36.87)	72.30 (68.92-78.18)
Pb 0	Cu	1.37 (0.88-1.86)	100 (98.56-100)
Pb 100		1.34 (1.09-1.60)	78.29 (74.37-82.22)
Pb 0	Zn	1.79 (1.36-2.22)	99.69 (98.59-100)
Pb 100		4.51 (3.87-5.14)	88.24 (87.03-89.45)

418

419 **Figure captions**

420 **Fig. 1** Dose/effect curves from experiment 1 showing % inhibition of β -glucosidase activity as a
421 function of Pb exposure concentrations expressed in mg/L (left graphs) and of TSS-normalized
422 Pb concentrations (right graphs) expressed in gPb/gTSS for each biofilm sample (1: site 1 biofilm
423 sample. 2: site 2 biofilm sample. 3: site 3 biofilm sample)

424

425 **Fig. 2** Dose/effect curves of biofilms exposed to 0, 1, 10 and 100 μ g/L Pb for three weeks
426 obtained by short-term toxicity tests with Pb (experiment 2). Curves represent the % inhibition of
427 β -glucosidase activity for each Pb concentration tested as a function of TSS-normalized Pb
428 concentrations (expressed in g_{Pb}/g_{TSS})

429

430 **Fig. 3** Dose/effect curves of Pb 0 and Pb 100 biofilms obtained by short-term toxicity tests with
431 Cu (a) and Zn (b). Curves represent the % inhibition of β -glucosidase activity for each metal
432 concentration tested as a function of TSS-normalized Pb concentrations (expressed in g_{Pb}/g_{TSS})

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