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## ► To cite this version:

A. Tlili, M. Maréchal, A. Bérard, B. Volat, B. Montuelle. Enhanced co-tolerance and co-sensitivity from long-term metal exposures of heterotrophic and autotrophic components of fluvial biofilms. *Science of the Total Environment*, 2011, 409 (20), p. 4335 - p. 4343. 10.1016/j.scitotenv.2011.07.026 . hal-00647096

**HAL Id: hal-00647096**

**<https://hal.science/hal-00647096>**

Submitted on 1 Dec 2011

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1 Enhanced co-tolerance and co-sensitivity from long-term metal  
2 exposures of heterotrophic and autotrophic components of fluvial biofilms

3

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24

25 **Abstract**

26 Understanding the interactive effects of multiple stressors on ecosystems has started to  
27 become a major concern. The aim of our study was therefore to evaluate the consequences of  
28 a long-term exposure to environmental concentrations of Cu, Zn and As on the pollution-  
29 induced community tolerance (PICT) of lotic biofilm communities in artificial indoor  
30 channels. Moreover, the specificity of the PICT was assessed by evaluating the positive and  
31 negative co-tolerance between these metals. Photosynthetic efficiency and substrate-induced  
32 respiration (SIR), targeting the autotrophic and heterotrophic communities respectively were  
33 used in short-term inhibition bioassays with Cu, Zn and As to assess sensitivities of pre-  
34 exposed biofilms to the metals tested. Diversity profiles of a phototrophic, eukaryotic and  
35 prokaryotic community in biofilms following the different treatments were determined and  
36 analyzed with principal component analysis. The results demonstrated that pre-exposure to  
37 metals induced structural shifts in the community and led to tolerance enhancements in the  
38 phototrophic and heterotrophic communities. On the other hand, whatever the functional  
39 parameter used (i.e. photosynthesis and SIR), communities exposed to Cu were more tolerant  
40 to Zn and vice versa. Furthermore, only phototrophic communities pre-exposed to As  
41 developed tolerance to Cu but not to Zn, whereas no co-tolerance between Cu and As was  
42 observed in the heterotrophic communities. Finally, phototrophic and heterotrophic  
43 communities exposed to Cu and Zn became more sensitive to As, reflecting a negative co-  
44 tolerance between these metals. Overall, our findings support the fact that although the mode  
45 of action of the different metals is an important driver for the structure and thus the tolerance  
46 of the communities, it appears that the detoxification modes are the most important factors for  
47 the occurrence of positive or negative co-tolerance.

48

49 Keywords: co-tolerance, ecological cost, river biofilm, metal toxicity.

## 50 **1. Introduction**

51 Considering the variability of aquatic environments, the multitude of contaminants and the  
52 inherent resistance and structural characteristics of communities, the assessment of the long-  
53 term impacts of chemical contamination of an environment is a complex issue. It is essential  
54 therefore to apply approaches that take into account biological and chemical complexity and  
55 variability of natural ecological systems to deal with the effects of metal pollution, and  
56 community ecotoxicology seems to be suitable for that (Clements and Rohr 2009). Lotic  
57 biofilms (attached microbial communities of autotrophic and heterotrophic, eukaryotic and  
58 prokaryotic populations) play a fundamental role in the aquatic trophic web and geochemical  
59 cycles (Battin et al, 2003). Indeed, biofilms are major sites for the uptake, processing and  
60 storage of dissolved organic carbon in lotic ecosystems. Because of their structural and  
61 functional complexity, biofilms integrate the effects of environmental conditions and are  
62 therefore considered as good early warning indicators of toxicants in aquatic ecosystems  
63 (Montuelle et al. 2010).

64 Pollution-induced tolerance could be defined as the capacity of an organism to cope with  
65 unfavourable environmental conditions resulting from the anthropogenic input of one or more  
66 pollutants into the environment. Previous studies have shown that gaining tolerance to  
67 toxicants could be the consequence of physiological acclimations of organisms during  
68 exposure, or of genetic adaptations (Bérard et al. 1998, Taylor and Feyereisen 1996). It is well  
69 recognized that the genetic adaptation is mainly due to the natural variability of the organisms'  
70 resistance toward pollutants (Clements and Rohr, 2009), which leads to the elimination of the  
71 most sensitive and the development of the most tolerant ones under chemical stress.

72 Based on these considerations, Blanck et al. (1988) proposed the concept of pollution-  
73 induced community tolerance (PICT) as an ecotoxicological tool that provides a good  
74 approach for environmental status characterization, useful not only for assessing immediate

75 impact but also for taking into account the contamination history of the ecosystem at the  
76 community level (Dorigo et al. 2004, 2010). The PICT is based on the fact that pollutants will  
77 exert a selection pressure and only the tolerant organisms will resist conferring a high  
78 tolerance to the whole community. Assessing the acquisition of tolerance is a method that  
79 should also try to establish a specific cause-effect relationship between one pollutant and its  
80 impact on biological communities. However, some issues still need to be clarified: Is it  
81 possible to target the specific effect of a given compound when modes of action and  
82 detoxification mechanisms are similar among chemicals thus leading to the same selection  
83 pattern? What are the consequences of enhanced tolerance to one stressor in the case of the  
84 occurrence of a new and different stressor?

85 In theory, PICT increase should be related to the selection pressure of one toxicant and  
86 thus reflects its presence in the ecosystem. However, this specificity is not absolute, and co-  
87 tolerance could occur. Co-tolerance is mainly caused by substances having a similar mode of  
88 action (Bérard et al. 2003, Molander 1991, Soldo and Behra 2000) or inducing a similar  
89 detoxification mechanism (Gustavson and Wängberg 1995). Nevertheless, co-tolerance is still  
90 insufficiently studied, especially since almost all studies that have addressed this issue in  
91 aquatic environments, were focused on the phototrophic component despite the importance of  
92 the heterotrophic one.

93 Furthermore, comparative ecotoxicology that is the basis of the PICT approach is also  
94 useful for understanding the consequences of enhanced tolerance. Acclimating or adapting to  
95 one set of environmental stressors may increase community susceptibility to novel stressors.  
96 The elimination of the most sensitive genotypes by pollution means the diminution of genetic  
97 variability and leads to a population specifically tolerant to that pollution, thus reducing the  
98 ability of the populations to cope with future disturbances (Kashian et al. 2007, Zuellig et al.  
99 2008). Despite the evident advantage of tolerance acquisition for maintaining an ecosystemic

100 process exposed to a given stress, it is clear that higher tolerance could also signify an  
101 alteration of community's efficiency to tolerate additional disturbances and therefore less  
102 ability to insure the ecosystem's recovery (Tobor-Kaplon et al., 2005).

103 In this study, we tested the hypothesis that depending on the targeted community, long-  
104 term exposure of lotic biofilm communities to a metal could lead to specific increased  
105 tolerance toward this metal and to an increase (or a decrease) of tolerance towards other  
106 metals to which they are naive. To confirm our hypothesis, we investigated the consequences  
107 of long-term exposure to environmentally realistic concentrations of Cu, Zn and As on  
108 biofilm community structure and their tolerance in artificial indoor channels. Various  
109 physiological parameters (targeting the autotrophic and heterotrophic communities) were used  
110 in short-term inhibition tests with Cu, Zn and As to assess sensitivities of pre-exposed  
111 biofilms to the metals tested.

112

## 113 **2. Material and methods**

### 114 **2.1. Sampling site**

115 The study was carried out on a small first-order stream (7 km long) (the Morcille  
116 river) which is subjected to strong agricultural pressure, essentially exerted by vineyards that  
117 occupy almost 80% of the 8.5 km<sup>2</sup> catchment area and is characterized by an increasing and  
118 permanent heavy metal gradient (Dorigo et al., 2010, Tlili et al. 2011) along the river. Cu, Zn  
119 and As are three of the metals most often detected. For the laboratory experiments, water  
120 carrying biofilm cells was collected from the Morcille River at the upstream, reference site.

### 121 **2.2. Experimental design**

122 To conduct this study, we used a total of 12 indoor glass channels (63 cm long, 11 cm  
123 wide and 4 cm deep) supplied separately with river water from the unpolluted reference site,  
124 which had been filtered through a 50- $\mu$ m mesh to remove most of the grazers. During all the

125 study, the experimental channels were used in recirculating mode: 5 L of water were  
126 recirculated at a rate of 1.2 L/min from a glass beaker located at the end of each channel  
127 through centrifugal pumps. All the glass beakers were placed in a refrigerated water bath for  
128 temperature control, and a tap at the head of each channel allowed similar flow regulation  
129 between all the experimental systems.

130 Biofilms colonized and grew for 5 weeks on artificial substrates (60 frosted glass disks  
131 of 1.5 cm<sup>2</sup> and 15 frosted glass slides of 18 cm<sup>2</sup>) installed horizontally in each channel. At the  
132 start of the experiment, the three channel replicates were contaminated separately with one of  
133 the three tested metals with a nominal concentration of 10 µg.L<sup>-1</sup> of Cu, added as CuSO<sub>4</sub>  
134 (Merck, 99% purity), 35 µg.L<sup>-1</sup> of Zn added as ZnSO<sub>4</sub> (Sigma-Aldrich GmbH, 98% purity)  
135 and 15 µg.L<sup>-1</sup> of As added as Na<sub>2</sub>HAsO<sub>4</sub> (Sigma-Aldrich GmbH; 98% purity). The three  
136 remaining channels were used as controls (no metals added). The toxicant (Cu, Zn and As)  
137 concentrations used in our study were similar to the maxima observed in Morcille river in late  
138 spring/early summer.

139 The water was kept at a temperature of 18°C (± 1°C), and exposed to a light intensity  
140 of 260 µmol m<sup>-2</sup>s<sup>-1</sup> under an 18:6 hr light/dark regime. These physical parameters were  
141 checked twice a week throughout the experiment. During the experiment, the channel water  
142 was replaced twice a week with 50-µm mesh-filtered water from the reference site to avoid  
143 nutritional deficiency, and depending on the experimental design, supplemented with Cu, Zn  
144 or As in order to maintain the nominal exposure.

### 145 **2.3. Biofilm sampling**

146 After five weeks of growth, biofilms were sampled and analyses performed: Total  
147 biomass (glass disks), bioassays (photosynthetic efficiency and substrate-induced respiration  
148 on glass disk and glass slides respectively) and chemical analysis (glass slides) were

149 performed immediately, whereas others (molecular and pigment analysis on big slides and  
150 small disk respectively) were performed subsequently on deep-frozen (-80°C) samples.

#### 151 **2.4. Physico-chemical analysis**

152 Parameters including oxygen (%), pH, conductivity, temperature and light were  
153 measured twice a week in each channel. In addition, 200 mL of water were collected from  
154 each channel to measure DOC (dissolved organic carbon),  $\text{PO}_4^{3-}$  and  $\text{SiO}_2$  concentrations just  
155 before and 1 hour after water renewal. Measurements were done following French standard  
156 operating procedures and protocols (AFNOR 1999). The laboratory which conducted the  
157 chemical analysis is accredited by the French Accreditation Committee (COFRAC)  
158 (accreditation number: 1-1238).

159 In order to measure the total dissolved Cu, Zn and As concentrations in the channels  
160 before and after each water renewal, 50 mL from each channel were filtered (Nylon  
161 Membrane Filters 0.2  $\mu\text{m}$ , Whatman, Maidstone, UK) and acidified with 1% of Supra pure  
162 nitric acid before storage at 4°C until analysis. Filtered samples were analyzed using  
163 inductively coupled plasma mass spectrometry (ICP-MS X Series II, Thermo Electron).

164 For quantification of total and internalized metals in biofilm, 4 pooled big glass slides  
165 (total surface area 72  $\text{cm}^2$ ) per channel were scraped using a polypropylene spatula and the  
166 biofilm suspended in 40 mL of 0.2  $\mu\text{m}$  Nuclepore-filtered water from the reference site. This  
167 suspension was divided into two fractions. Fraction one (20 mL) was treated with 320  $\mu\text{L}$  of  
168 4.0 mM EDTA (final concentration) to quantify internalized Cu, Zn and As, and fraction two  
169 was used to quantify total in-biofilm Cu, Zn and As. Biofilm suspensions were then treated  
170 following Tlili et al. (2010). Briefly, biofilms were filtered (cellulose nitrate 0.45  $\mu\text{m}$   
171 membrane, Millipore) and dried for 24h at 50°C. Dry samples were digested with 3 ml of  
172 concentrated nitric acid (Supra pure) and 1 ml of 30% hydrogen peroxide in a high-  
173 performance microwave oven (Milestone, Ethos sel) and 25 mL Milli-Q water was added to



174 dilute the acid concentrate. The water samples were analyzed following the same procedure as  
175 for total dissolved metal concentrations in water. The percentage internalization for each  
176 metal was determined by dividing the concentration of internalized metal in cells (after EDTA  
177 treatment) by the total concentration measured in the biofilm matrix (without EDTA  
178 treatment).

## 179 **2.5. Total and phototrophic biomass**

180 The organic matter content in 3 pooled small disks (4.5 cm<sup>2</sup> / channel) was calculated  
181 as described in Tlili et al. (2008). Results are expressed as g.m<sup>-2</sup>. Chlorophyll-*a* content in  
182 biofilm was considered to be an indicator of the phototrophic biomass (Bonin and Travers,  
183 1992) and was quantified by HPLC analysis. The chlorophyll-*a* was quantified using external  
184 calibrations of standard chlorophyll-*a* (C55H72MgN4O5, Carl Roth GmbH & Co). Final  
185 concentrations are given as µg.cm<sup>-2</sup> (see 2.7.1 for analytical details).

## 186 **2.6. Biofilm function analysis**

### 187 2.6.1. Photosynthetic efficiency

188 In order to measure the maximum photosynthetic efficiency, we used the Pulse of  
189 Amplitude Modulated fluorometry (PhytoPAM, Heinz Walz GmbH, Germany) technique.  
190 Three small disks per channel were sampled and after 30 minutes of dark adaptation, they  
191 were submitted to a single saturation pulse allowing the measurement of the maximum  
192 quantum yield ( $Y_{II_{665nm}}$ ), which is an indicator of the photosynthetic efficiency, as:

$$193 \quad Y_{II_{665nm}} = \frac{F_m - F_0}{F_m}$$

194 where  $F_m$  is the maximum fluorescence after the saturation pulse and  $F_0$  is the steady-state  
195 fluorescence.

### 196 2.6.2 Substrate-induced respiration

197 The substrate-induced respiration (SIR) of the heterotrophic biofilm communities was  
198 measured using the MicroResp<sup>TM</sup> method according to Tlili et al. (2011). MicroResp<sup>TM</sup>

199 system is a colorimetric method well described by Campbell et al. (2003) and consists of two  
200 microplates (96 wells) placed face to face. One of these is a deep-well microplate (1.2 mL  
201 capacity, 96-deep-well microplate, NUNC) in which each well contains the biofilm sample  
202 with the carbon source, and the second microplate (detection microplate) contains a pH  
203 indicator dye that changes proportionally to the amounts of CO<sub>2</sub> released by heterotrophic  
204 communities.

205 Biofilm was scraped off substrata (4 pooled glass slides / channel) and suspended in  
206 60 mL of 0.2 µm Nuclepore-filtered water from the reference site. This suspension was  
207 divided into two fractions: the first (30 mL) was used for the SIR and basal respiration  
208 measurement and the second (30 mL) for the SIR bioassays (see 2.6.3).

209 Briefly, 500 µL of biofilm suspensions were distributed in the deep-wells and 30 µL of  
210 glucose solution (carbon source) were added with final concentration of 6.2 mg of C per mL.  
211 After positioning the detection microplate, the system was sealed and incubated for 15 hours  
212 in the dark at room temperature (20 ± 1 °C). CO<sub>2</sub>-trapped absorbance was measured at  
213 570 nm immediately before sealing to the deep well plate, and after 15 h incubation. The  
214 results were expressed in µg CO<sub>2</sub>.mg<sup>-1</sup>AFDW.h<sup>-1</sup>. In addition to the SIR measurements, the  
215 basal respiration (without glucose addition) was measured for each biofilm. Results of  
216 induced-respiration with glucose were normalized by the basal respiration to target only the  
217 heterotrophic communities (Tlili et al. 2011).

### 218 2.6.3. Biofilm phototrophic and heterotrophic sensitivity measurements to Cu, Zn and As

219 To investigate sensitivities (pollution-induced community tolerance, PICT) to Cu, Zn  
220 and As, acute toxicity tests with pre-exposed and control biofilm samples from all the  
221 channels were performed. Photosynthetic efficiency and substrate-induced respiration  
222 (targeting phototrophic and heterotrophic communities respectively) were used as functional  
223 parameters in these bioassays.

224 Stock solutions containing  $2.10^{-1}$  M Cu ( $\text{CuSO}_4$ ; Merck high-purity grade) or 2 M Zn  
225 ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; Sigma high purity grade) or  $2.10^{-1}$  M As ( $\text{NaHAsO}_4$ ; Sigma high-purity grade)  
226 were prepared in milli-Q water and stored at  $4^\circ\text{C}$  prior to dilution in the test vessels. Semi-  
227 logarithmic series of concentrations were freshly prepared by serial dilutions of the stock  
228 solutions in  $0.2 \mu\text{m}$ -filtered water from the reference site. For photosynthetic bioassays (3  
229 blanks and 3 replicates for each of the 6 increasing concentrations), final test concentrations  
230 ranged from 0 to  $2.10^3 \mu\text{M}$  for Cu, from 0 to  $6.10^3 \mu\text{M}$  for Zn and from 0 to  $2.10^5 \mu\text{M}$  for As,  
231 whereas for SIR bioassays (3 blanks and 3 replicates for each of the 9 increasing  
232 concentrations) they ranged from 0 to  $2.10^4 \mu\text{M}$  for Cu and As, and from 0 to  $2.10^5 \mu\text{M}$  for  
233 Zn.

234 - *Short-term photosynthetic bioassays*: Biofilms from each channel (small glass disk)  
235 were exposed to increasing concentrations of Cu, Zn and As during 2, 4 and 2.5 hours  
236 respectively, under the same light intensity and temperature conditions as their growth.  
237 Measurements were performed using a PhytoPAM fluorometer and the relative inhibition of  
238 photosynthetic efficiency at 665 nm in relation to the control was calculated using a dose-  
239 response relationship to measure the  $\text{EC}_{50}$  for each bioassay. The durations of the bioassays  
240 with the different metals were determined during a previous step to know the minimum of  
241 incubation time allowing inhibition of photosynthetic efficiency by 50%.

242 - *Short-term SIR bioassays*: Biofilm suspensions were distributed in deep wells  
243 ( $500 \mu\text{L}$  per well), to which we added  $50 \mu\text{L}$  of the metal solutions (increasing  
244 concentrations). The microplate was then pre-incubated in the dark at room temperature for 3  
245 hours (to cope with artefacts causing chemical release of  $\text{CO}_2$ ), and then  $30 \mu\text{L}$  of the glucose  
246 solution ( $120 \text{mg} \cdot \text{mL}^{-1}$ ) was added to each well and the detection microplate was positioned.  
247 Incubation and  $\text{CO}_2$  measurements were processed afterwards as described above (section

248 2.6.2). EC<sub>50</sub> values were calculated from dose-response curves by plotting biofilm CO<sub>2</sub>  
249 production at each metal concentration.

## 250 **2.7. Biofilm structure analysis**

251 2.7.1. Phototrophic pigment identification by high-performance liquid chromatography  
252 (HPLC)

253 Three pooled glass disks (4.5 cm<sup>2</sup> / channel) were selected and processed for HPLC  
254 pigment analysis as described in Tlili et al. (2008). Identification of the different pigments  
255 was performed depending on their specific retention times and absorption spectrum using  
256 DAD according to SCOR (Jeffrey et al., 1997). A table was constructed (with samples as  
257 rows and pigments as columns) by taking into account the relative abundance of each pigment  
258 in a given sample (expressed as a percentage of the sum of the area of all the pigments in a  
259 sample). In addition, a quantitative method from a calculation model based on published  
260 ratios for cultures containing a mixture of different algae (Wilhem et al. 1991) was used to  
261 determine the relative abundance of diatoms, cyanobacteria and green algae from their  
262 specific pigment signatures (fucoxanthin, zeaxanthin and lutein, respectively). Final  
263 concentrations are given as µg.cm<sup>-2</sup>.

264 2.7.2. Eukaryotic and prokaryotic community structure assessed by denaturing gradient gel  
265 electrophoresis (DGGE)

266 Determination of the eukaryotic and prokaryotic composition was performed  
267 according to Dorigo et al. (2007). Briefly, biofilm from each channel was scraped off 1 big  
268 glass substratum, then centrifuged at 14,000 g for 30 minutes at 4°C and the supernatant was  
269 removed. Nucleic acid was extracted from the biofilm pellets using the DNAeasy Plant kit  
270 (QIAGEN) following the manufacturer's instructions. PCR amplification of eukaryotic 18S  
271 rRNA gene fragments, and prokaryotic 16S rRNA gene fragments, and their DGGE analysis  
272 were performed according to Tlili et al. (2008). After migration, separated PCR products were

273 stained for 45 min in the dark with SYBRGold (Molecular probes), visualized on a UV  
274 transilluminator (Clavision), and photographed (Scion Corporation camera). Prokaryotic-  
275 and eukaryotic-DGGE profiles were analyzed by the GelCompar II software (Applied Math  
276 NV) leading to a matrix based on the quantification of relative band intensities. Furthermore,  
277 diversity based on the Shannon indexes was calculated for all the samples.

## 278 **2.8. Statistical data processing**

279  $EC_{50}$  values were calculated by means of the Regtox model (E. Vindimian,  
280 <http://eric.vindimian.9online.fr/>). The tolerance or sensitivity ratios (R) ( $R > 1$  = induced  
281 tolerance and  $R < 1$  = induced sensitivity) were calculated by dividing the  $EC_{50}$  (pre-exposure)  
282 by the corresponding  $EC_{50}$  (control). for phototrophic (based on photosynthesis bioassays)  
283 and heterotrophic (based on SIR bioassays) biofilm communities sampled from the control  
284 channels and from contaminated channels with Cu, Zn and As.

285 The effects of long-term exposure to each metal on total and % of internalization of  
286 each metal into the biofilm matrices ( $n = 3$ ), AFDW ( $n = 3$ ), algal biomass ( $n = 3$ ),  
287 photosynthesis and SIR  $EC_{50}$ 's values ( $n = 3$ ), SIR ( $n = 3$ ), photosynthetic efficiency ( $n = 3$ ),  
288 and Shannon index ( $n = 3$ ) values were tested by factorial 1-way ANOVA on XLSTAT  
289 software (2009 version). If a main effect was significant, the ANOVA was followed by a  
290 Tukey-HSD test.

291 Structural data including DGGE - 18S and 16S rRNA (relative band intensities), and  
292 phototrophic pigment identification (relative abundances normalized by chlorophyll-*a*) were  
293 used to determine eukaryotic, prokaryotic, and phototrophic pigment diversities respectively.  
294 Before their use in the analysis, data were  $\log_{10}(x + 1)$  transformed and then submitted to  
295 detrended correspondence analysis (DCA). The lengths of all the gradients (1.399; 0.861 and  
296 0.789 for eukaryotes; prokaryotes and phototrophs respectively) indicated that linear methods

297 were appropriate for all the structural endpoints. Principal component analyses (PCA) were  
298 therefore performed, using CANOCO software version 4.5 (ter Braack and Smilauer 1998).

299

## 300 **2. Results**

### 301 **2.1. Physico-chemical data**

#### 302 **Table 1**

303 Physico-chemical parameters from the reference site and from channel water samples  
304 are given in Table 1. Oxygen concentrations and pH were quite similar between the different  
305 treatments, whereas in all the channels compared to the reference site, a decrease in  
306 phosphates and silicates and an increase in DOC were detected. Control channels presented  
307 lower conductivity than Cu-, Zn- and As-contaminated channels.

308 The Cu, Zn and As concentrations in the water of the control channels were  $2.1 \pm 0.3$   
309  $\mu\text{g.L}^{-1}$ ,  $7.6 \pm 2.4 \mu\text{g.L}^{-1}$  and  $1.2 \pm 0.1 \mu\text{g.L}^{-1}$  respectively. In the water of Cu-contaminated  
310 channels, Cu concentration was  $10.3 \pm 1.7 \mu\text{g.L}^{-1}$  whereas Zn and As concentrations were  
311 similar to those of the controls ( $6.8 \pm 2.6$  and  $1.2 \pm 0.2 \mu\text{g.L}^{-1}$  respectively). In the Zn-  
312 contaminated channels, Zn concentration was  $28 \pm 6.7 \mu\text{g.L}^{-1}$  and no difference with the  
313 control channels for Cu ( $2.6 \pm 0.7 \mu\text{g.L}^{-1}$ ) or As ( $1.2 \pm 0.2 \mu\text{g.L}^{-1}$ ) concentrations was  
314 observed. Arsenic concentrations in the water of As-contaminated channels was  $15.8 \pm 1.5$   
315  $\mu\text{g.L}^{-1}$  whereas Cu and Zn concentrations were similar to those measured in the control  
316 channels ( $2.5 \pm 0.8$  and  $7.0 \pm 3.1 \mu\text{g.L}^{-1}$  respectively).

#### 317 **Table 2**

318 Cu, Zn and As concentrations accumulated (total and % internalization) in biofilms are  
319 summarized in Table 2. The amounts of total Cu in the Cu-exposed biofilms were higher than  
320 those measured in the controls, and the same trends were observed for Zn and As  
321 concentrations in the Zn- and As- exposed biofilms respectively (Tukey test  $p < 0.05$ ). In the

322 Cu- and Zn- exposed biofilms, the % of internalization of Cu and Zn were significantly lower  
323 than in the control biofilms (Tukey test  $p < 0.05$ ), whereas no difference was observed for the  
324 % of internalization of As, in comparison with the control biofilms. For the As-exposed  
325 biofilms, no significant difference was observed in the % internalization of Cu, Zn and As, in  
326 comparison with the control biofilms (Tukey test  $p > 0.05$ ).

327

## 328 **2.2. Biomass parameters** (Table. 3)

### 329 **Table 3**

330 There was no significant effect of Cu, Zn and As exposure on the AFDW (1-way  
331 ANOVA,  $p > 0.05$ ). Chlorophyll-*a* concentrations were similar between Cu-, Zn-exposed and  
332 control biofilms (Tukey test  $p > 0.05$ ), whereas only pre-exposure to As induced a significant  
333 and sharp decrease in the chlorophyll-*a* concentrations (Tukey test  $p < 0.001$ ).

## 334 **2.3. Biofilm activities**

### 335 2.3.1. Photosynthetic efficiency (Table. 3)

336 No significant difference was observed for the photosynthetic efficiency between Cu-  
337 and Zn-exposed and control biofilms (Tukey test  $p > 0.05$ ). In contrast, pre-exposure to As  
338 induced a significant decrease in the photosynthetic efficiency in comparison with the Cu-  
339 and Zn-exposed and control biofilms (Tukey test  $p < 0.05$ ).

### 340 2.3.2. Heterotrophic SIR (Table. 3)

341 Exposure to metals had a significant effect on biofilm SIR (1-way ANOVA,  $p <$   
342  $0.001$ ). Cu-, Zn- and As-exposed biofilms were characterized by lower SIR than the controls  
343 (Tukey test  $p < 0.001$ ). No significant SIR differences were observed between biofilms from  
344 the different treatments (Tukey test  $p > 0.05$ ).

## 345 **2.4. Assessment of biofilm sensitivities to Cu, Zn and As**

### 346 **Table 4**

347 2.4.1. Photosynthetic efficiency bioassay (Table. 4)

348 - *Specific tolerance measurements*: Overall, in comparison with the control biofilms,  
349 phototrophic communities pre-exposed to Cu, Zn or As showed a significantly higher  $EC_{50}$   
350 values of Cu ( $R = 2.65$ ; Tukey test  $p < 0.01$ ), Zn ( $R = 2.64$ ; Tukey test  $p < 0.001$ ) or As ( $R =$   
351  $224.51$ ; Tukey test  $p < 0.0001$ ) respectively.

352 - *Cross sensitivities*: Pre-exposure to Cu induced enhanced tolerance of the  
353 phototrophic communities to Zn ( $R = 2.96$ ) but an increase in their sensitivity to As ( $R =$   
354  $0.24$ ). Likewise, biofilms pre-exposed to Zn showed an increased tolerance to Cu ( $R = 2.06$ )  
355 and also a higher sensitivity to As ( $R = 0.51$ ). Pre-exposure to As led to enhanced tolerance to  
356 Cu ( $R = 2.88$ ) but not to Zn ( $R = 1$ ).

357 2.4.2. SIR bioassay (Table. 4)

358 - *Specific Tolerance measurements*: Pre-exposure to Cu induced a significant increase  
359 of the heterotrophic communities' tolerance to Cu itself ( $R = 2.12$ ; Tukey test  $p < 0.01$ ).  
360 Similarly, heterotrophic communities pre-exposed to Zn or to As displayed a higher tolerance  
361 to Zn ( $R = 11.15$ ; Tukey test  $p < 0.001$ ) and As ( $R = 3.44$ ; Tukey test  $p < 0.001$ ) respectively.

362 - *Cross sensitivities*: In addition to their enhanced tolerance to Cu, heterotrophic  
363 communities pre-exposed to Cu also increased their tolerance to Zn ( $R = 2.90$ ) and similarly,  
364 communities pre-exposed to Zn showed an increase of their tolerance to Cu ( $R = 2.23$ ). On  
365 the other hand, pre-exposure of heterotrophs to Cu and Zn led to an increase of their  
366 sensitivity to As ( $R = 0.24$  and  $0.61$  respectively). Finally, biofilms pre-exposed to As, did not  
367 develop any tolerance or sensitivity to Cu and Zn ( $R = 1$ ).

368 **2.5. Structural analysis**

369 2.5.1. Phototrophic pigment analysis

370 **Fig.1**



371 The relative abundance of diatoms, cyanobacteria and green algae was influenced by  
372 their exposure to the different metals (1-way ANOVA,  $p < 0.001$ ,  $p < 0.0001$  and  $p < 0.0001$   
373 respectively) (Fig.1). Exposure to Cu, Zn or As induced a same and significant decrease in the  
374 abundance of diatoms in comparison with the control (Tukey test  $p < 0.05$ ). On the other  
375 hand, only exposure to As led to a large and significant increase of the abundance of  
376 cyanobacteria (Tukey test  $p < 0.0001$ ). Finally, green algae were significantly more abundant  
377 in the Cu- and Zn- exposed biofilms than in the controls (Tukey test  $p < 0.05$ ), whereas they  
378 disappeared completely from the As-exposed biofilms (Tukey test  $p < 0.0001$ ).

### 379 **Fig. 2**

380 A total of 12 pigments were detected in all samples collected. Among them only 9  
381 (which accounted for at least 2 % in two samples) were included in the PCA (Fig.2). The two  
382 first axes (PC1 and PC2) of the PCA accounted for more than 88 % of the total variability.  
383 Control biofilms were separated on PC1 from Cu-, Zn- and As- exposed biofilms, whereas  
384 PC2 separated As-exposed biofilms from the other ones. PC1 was positively correlated to  
385 diadinoxanthin, violaxanthin and carotene, and negatively correlated to the dinoxanthin  
386 whereas PC2 was positively correlated to zeaxanthin, and negatively correlated to lutein.

### 387 2.5.2. Eukaryotic diversity and richness

### 388 **Table 5**

389 Table.5 summarizes the Shannon indexes (H) calculated for the eukaryotic  
390 community. A total of 41 OTU's were detected after the DGGE analysis. Overall, exposure to  
391 metals had a significant effect on "H" (1-way ANOVA,  $p < 0.0001$ ). Eukaryotic communities  
392 in the control biofilms showed a significantly higher "H" than Cu-, Zn- and As-exposed ones  
393 (Tukey test  $p < 0.0001$ ), and no significant difference was observed between the different  
394 exposed biofilms (Tukey test  $p > 0.05$ ).

### 395 **Fig. 3**

396 PCA was applied to the bands' relative intensities data for samples from the different  
397 channels (Fig.3). The two first axes (PC1 and PC2) explained more than 77 % of the total  
398 variability of eukaryotic diversity. PC1 (39.68%) separated control biofilms very clearly from  
399 Cu- Zn- and As-exposed ones, whereas Zn- and Cu-exposed biofilms were separated from  
400 control and As-exposed biofilms on PC2 (37.79 %). Overall, in these two projections, there  
401 was very low variability between the three replicates in each channel.

### 402 2.5.3. Prokaryotic diversity and richness

403 Table.5 summarizes the Shannon index (H) calculated for the prokaryotic community.  
404 Considering overall the biofilm samples, 43 different OTU's were detected after the DGGE  
405 analysis. Exposure to Cu, Zn and As induced a significant decrease in the prokaryotic "H" in  
406 comparison with the control biofilms (1-way ANOVA,  $p < 0.001$ ). As-exposed biofilms were  
407 characterized by the lowest "H" (Tukey test  $p < 0.001$ ), whereas no significant difference was  
408 observed between Cu- and Zn-exposed biofilms (Tukey test  $p > 0.05$ ).

409

### 410 **Fig. 4**

411 PCA performed with the relative band intensity data of samples from different  
412 channels (Fig. 4), showed that the first two axes (PC1 and PC2) accounted for more than 69  
413 % of the total variability of the prokaryotic structure. PC1 (37.7 %) separated the As-exposed  
414 biofilms very clearly from the control, the Cu- and Zn-exposed biofilms. The second axis,  
415 PC2 (31.36 %), separated control and Cu-contaminated channel samples from those taken  
416 from the Zn- and As-contaminated channels. The variability between the three replicates in  
417 each channel was also very low.

418

## 419 **4. Discussion**

420 The concentrations of dissolved nutrients, especially  $\text{PO}_4^{3-}$  and  $\text{SiO}_2$  (phosphates are  
421 used by all organisms in biofilm, and silicates are principally used by diatoms) at the  
422 sampling dates in all the channels were very low compared to the initial values (reference site  
423 water), suggesting that a rapid uptake of nutrients had occurred in our experimental  
424 conditions. The DOC concentrations, however, increased in all the channels, suggesting  
425 autochthonous organic matter production by algae. Our physico-chemical measurements also  
426 showed a similar increase of the conductivity values in the contaminated channels but not in  
427 the control one. These results were expected since the addition of metal ions induces an  
428 increase in water conductivity (Guasch et al. 2002). Indeed, conductivity corresponds to the  
429 presence of anions and cations in water, and metal inputs in the water generally lead to the  
430 increase of these anions and cations (according to the metal speciation) and consequently to  
431 increased conductivity.

432 *(i) Exposure to one metal enhances phototrophic and heterotrophic tolerance to this metal but*  
433 *also to others*

434 Some trace metals such as Cu or Zn play indispensable roles in cell growth and  
435 maintenance of metabolic functions (De Filippis and Pallaghy 1994). Nevertheless, at high  
436 concentrations these trace metals could become toxic (Soldo and Behra 2000). On the other  
437 hand, some metals such as As are not required for cell growth and are considered as toxic  
438 even at low doses (Hughes and Poole, 1986). Our investigations confirmed the higher toxicity  
439 of As than of Cu and Zn, particularly on the phototrophic biofilm component. Indeed they  
440 showed that even though there was no difference in total biofilm biomass between the  
441 different channels (including the controls), the chlorophyll-*a* content decreased sharply in the  
442 biofilms exposed to As, unlike those that were exposed to Cu or Zn, in comparison with the  
443 control ones. In addition, the same trend was observed for the photosynthetic activity  
444 measurement since only biofilms exposed to As showed lower photosynthesis than the control

445 ones, unlike biofilms exposed to Cu or Zn. It is well known that to counteract the toxic effects  
446 of metals, phototrophic cells have evolved various mechanisms. Previous studies showed that  
447 in the presence of Cu or Zn, cells can change their membrane permeability, thus decreasing  
448 metal internalization into cells, which is driven chemiosmotically (Soldo 2005, Serra et al.  
449 2009). Nevertheless, As internalization is independent of the membrane permeability, since  
450 As is transported into cells actively by two phosphate transporters (Rosen 1996). In our  
451 experiment, in comparison with the control biofilms, pre-exposure to Cu and Zn caused a  
452 decrease in the percentage of internalization of these two metals, but this was not the case for  
453 As. In contrast, pre-exposure to As did not show any significant effects on the percentage of  
454 internalization of Cu, Zn or As. These results could therefore explain the negative effects of  
455 As on the phototrophic biofilm communities and the lack of effect of Cu and Zn on the  
456 biological parameters measured. Conversely, all three metals tested (i.e., Cu, Zn and As)  
457 inhibited the heterotrophic activity, based on the SIR measurements, suggesting that only  
458 phototrophs could modulate their membrane permeability as a resistance mechanism to metal  
459 and not the heterotrophic communities.

460 In spite of the negative effects, observed on the algal biomass, photosynthetic activity  
461 and SIR, enhanced tolerance to the tested metal was observed for all the pre-exposed biofilms.  
462 Indeed, over 2-fold increases in the level of tolerance to Cu were observed in phototrophic  
463 and heterotrophic communities that were pre-exposed to Cu, when these communities were  
464 compared to the controls. Similarly, pre-exposure to Zn also induced more than 2- and 11-fold  
465 enhanced tolerance to this metal in phototrophs and heterotrophs respectively, in comparison  
466 with the control communities. On the other hand, As exposure led to a 224-fold higher  
467 tolerance to As in phototrophic communities than in control communities, whereas tolerance  
468 was enhanced 3-fold in heterotrophs. According to the PICT concept, our results could be  
469 related to the fact that these metals provided selection pressure on phototrophs and

470 heterotrophs, inducing a shift in the community structures with the emergence of the most  
471 tolerant species (Tlili and Montuelle, 2011). All the structural endpoints in our study,  
472 confirmed these assumptions, showing a divergence between all exposed biofilms and the  
473 controls. Indeed, HPLC analysis for example, showed that phototrophic communities were  
474 structured by exposure to the metals. Overall, even if diatoms were dominant in all the  
475 sampled biofilms, Cu, Zn and As exposure induced a decrease in their relative abundance in  
476 comparison with the control biofilms. Conversely, the relative abundance of green algae  
477 increased after exposure to Cu and Zn and dramatically decreased after As exposure, and the  
478 relative abundance of cyanobacteria increased after exposure to As. Numerous studies have  
479 also reported the restructuring effect of metals on the phototrophic biofilm communities, with  
480 the dominance of green algae in Cu or Zn exposed biofilms (Genter et al. 1987, Serra et al.  
481 2009, Tlili et al. 2010) or the dominance of cyanobacteria after As-exposure (Thiel 1988).  
482 Multivariate analysis (PCA) of eukaryotic and prokaryotic diversity and phototrophic  
483 pigments analysis also showed that the pre-exposed biofilms were very different from the  
484 control ones.

485         Increases in levels of tolerance to metals other than those that were originally added to  
486 the channels were also found for the phototrophic and heterotrophic communities when they  
487 were compared with communities obtained from control channels, indicating co-tolerance  
488 induction. Indeed, whatever the endpoint used for EC<sub>50</sub> measurement (i.e., photosynthesis or  
489 SIR), Cu-exposed biofilms showed enhanced tolerance to Zn and vice versa, indicating  
490 common tolerance mechanisms for Cu and Zn and for both of phototrophic and heterotrophic  
491 communities. Gustavson and Wängberg (1995) or Soldo and Behra (2000) observed such  
492 enhanced tolerance to Zn based on photosynthesis bioassays with phytoplankton and  
493 periphytic communities respectively after their exposure to Cu. In a soil study, Diaz-Ravina et

494 al. (1994) showed that Zn-exposed or Cu-exposed bacterial communities were characterized  
495 by a higher tolerance to both Cu and Zn.

496 Two hypotheses could explain the co-tolerance induction between Cu and Zn: the first  
497 is based on similar modes of action leading to the same selection pressure and thus to the  
498 same tolerant species, and the second hypothesis is related to similar modes of detoxification,  
499 not necessarily involving the same species.

500 Our PCA results for phototrophic pigment and eukaryotic diversity, showed that Cu  
501 and Zn exposed biofilms were very similar (following PC2 axis), confirming the hypothesis  
502 that Cu and Zn exerted a similar selection pressure on these communities. On the other hand,  
503 PCA analysis of the prokaryotic diversity, showed that Cu and Zn exposure did not select a  
504 similar community (following PC2 axis), in spite of the occurrence of co-tolerance (based on  
505 SIR bioassays). It is well established that even though the exact target sites might differ  
506 (Stratton 1987), Cu and Zn share the same modes of action: at high concentrations they could  
507 inhibit the photosynthesis reaction by blocking electron transfer at PSI and PSII levels, and  
508 consequently stop oxygen release and CO<sub>2</sub> fixation (Serra et al. 2009, Ivorra et al. 1999).  
509 Moreover, Zn and Cu could act negatively on the respiratory activities (Nalewajko and  
510 Olaveson 1994) or on some enzymes (Hughes and Poole 1989). Given these similar modes of  
511 action, pre-exposure to Cu or Zn could therefore promote the development of the same  
512 tolerant organisms (i.e. phototrophs) or different organisms but possessing the same resistance  
513 mechanisms (i.e. heterotrophs) against these two metals. Overall, we can assume that the  
514 occurrence of co-tolerance between Cu and Zn is modulated by similar modes of action and  
515 detoxification for phototrophic communities, and by similar detoxification modes for  
516 heterotrophic communities.

517 Interestingly, exposure to As increased the tolerance of phototrophic communities to  
518 As and Cu and not their tolerance to Zn, while exposure to Cu or Zn did not induce any

519 enhanced tolerance to As. Our findings are in accordance with those obtained by Blanck and  
520 Wängberg (1991) which showed that As specifically selected traits with increased As  
521 tolerance in phytoplankton communities, but at the same time also selected species with a  
522 large genetic variability that allowed more general tolerance increases. Unlike Cu and Zn  
523 exposure, exposure to As was shown here to increase the relative abundance of cyanobacteria.  
524 We indicated previously that As may be transported into cells by phosphate carriers and can  
525 then interfere with many enzymatic reactions involving phosphate (Rosen, 1996). According  
526 to Thiel (1988), cyanobacteria have the ability to discriminate between phosphate and As and  
527 are therefore tolerant to this metal. Moreover, cyanobacteria are also tolerant to Cu  
528 (Barranguet et al. 2000, Roussel et al. 2007). This could explain the enhanced tolerance of  
529 phototrophs to Cu after exposure to As.

530         Conversely, pre-exposure to As in our study, did not enhance tolerance (based on SIR  
531 bioassays) of heterotrophic biofilm communities to Cu or Zn. One of the hypotheses that  
532 could explain these results is related to the selection pressure that could be exerted differently  
533 by the metals tested, leading to completely different heterotrophic communities. Indeed,  
534 analysis of prokaryotic diversity, showed that Cu, Zn and As exposure led to dissimilar  
535 prokaryotic communities. However, this assumption does not seem to be as important for  
536 explaining our results, because in this case, no co-tolerance should be observed between Cu  
537 and Zn for the heterotrophic communities, which was not the case in our study. A reasonable  
538 interpretation of our findings may be therefore the specificity of the mechanisms involved in  
539 As detoxification by heterotrophic communities. This may explain the co-tolerance observed  
540 between Cu and Zn, and not with As, for heterotrophic communities (e.g. bacteria).

541         Overall, our findings suggest that the occurrence of co-tolerance does not necessarily  
542 follow the same patterns, since it depends on various but interlinked factors such as (i) the  
543 metal to which communities were chronically exposed, (ii) the mode of action of the metals,

544 (iii) the detoxification mechanisms implemented by cells, and (iv) the microbial community  
545 targeted (e.g., heterotrophs, heterotrophs). Indeed, we showed in this study that, although  
546 contamination with different metals could select for Cu phototrophic-tolerance, the tolerance  
547 to Cu did not automatically lead to tolerance to the other metals. Furthermore, the ubiquitous  
548 co-tolerance to Cu that was observed in phototrophs disappeared in the case of heterotroph  
549 tolerance. In the light of our results and those of other studies (for example Diaz-Ravina et al.  
550 1994, Soldo and Behra 2000), Cu may be therefore proposed as a “model” for a given class of  
551 toxicant (i.e. heavy metals) in a preliminary PICT approach, aiming to limit the number of  
552 suspect pollutants in ecotoxicological assessment. Afterward, a combination of various  
553 tolerance measurements, based on other activities such as SIR, could allow us to refine our  
554 investigations into tolerance mechanisms.

555 *(ii) Enhanced tolerance to one metal increases susceptibility to others*

556 The PICT approach was employed to demonstrate that the community restructuring  
557 was a direct result of contaminant exposure, but few studies have investigated the effects of  
558 community restructuring on their susceptibility to other stressors in aquatic ecosystems. As  
559 described above, the evolution of tolerance to contaminants may provide benefits via co-  
560 tolerance to other stressors. In our study, when biofilms were exposed to Cu and Zn, we  
561 observed enhanced tolerance of phototrophic and heterotrophic communities to these two  
562 metals. Nevertheless, we also observed that these biofilms tolerant to Cu and Zn became more  
563 sensitive to As whatever the targeted community (i.e., heterotrophs or phototrophs) For  
564 example, it has been shown that microbial communities pre-exposed to metals are more  
565 sensitive to UV-B than communities not pre-exposed (Clements et al. 2008, Kashian et al.  
566 2007, Zuellig et al. 2008).

567 At the community level, the development of enhanced tolerance could be related to the  
568 reduction of the global genetic variation in the community due to the strong selective pressure



569 exerted by stressors (e.g. metals) and the elimination of sensitive species, which is the basis of  
570 the PICT concept (Kashian et al. 2007). In our study, the calculation of the Shannon index for  
571 eukaryotic and prokaryotic communities, confirmed the loss of species diversity after  
572 exposure to Cu and Zn, in comparison to control biofilms. In many respects, loss of species  
573 diversity is comparable to reduced genetic variation, with similar implications for the  
574 ecological functions of ecosystems (Taylor and Feyereisen 1996). Indeed, Paine et al. (1998)  
575 stipulated that when an assemblage is already maintained in an altered state (i.e. metal pre-  
576 exposure in our case), occurrence of superimposed disturbance may lead to long-term  
577 alteration of the community state, therefore affecting the ecosystem's potential for recovery.  
578 Furthermore, community resistance to additional stressors may depend on tolerances induced  
579 by pre-exposure to stress. Initial exposure to a stressor combined with positive species co-  
580 tolerance should reduce the impacts of other stressors, which Vinebrooke et al. (2004) terms  
581 “stress-induced community tolerance”. Inversely, initial exposure to a stressor combined with  
582 negative species co-tolerance should increase the impacts of other stressors, which  
583 Vinebrooke et al. (2004) terms “stress-induced community sensitivity”.

584 In-depth study of the potential biological consequences associated to enhancing  
585 tolerance to stressors, may therefore help in understanding the role of diversity and positive or  
586 negative species co-tolerance in the maintenance of ecosystem function and the rate of  
587 recovery in communities after disturbance removal (Clements and Rohr 2009; Vinebrooke et  
588 al., 2004).

589

## 590 **5. Conclusion**

591 Multiple stressors in natural aquatic systems could make the establishment of a specific link  
592 between a given toxicant and its effects on organisms difficult, even if the concept of  
593 pollution-induced community tolerance (PICT) (Blanck et al. 1988) is proposed as a tool to

594 overcome this drawback. Our results support the PICT concept, since we showed that  
595 exposure to a given metal led to structural shifts of the community and therefore to enhanced  
596 tolerance to this metal. Nevertheless, we also showed that, depending on the chronic stressor  
597 and the targeted community (i.e. phototrophs or heterotrophs), the consequences of enhanced  
598 tolerance were various, since tolerance increase to one metal was positively (i.e. co-tolerance)  
599 or negatively correlated (i.e. co-sensitivity) to tolerance to other metals. In the light of our  
600 results, selection pressures exerted by metal exposures and therefore the selected species may  
601 modulate co-tolerance and co-sensitivity. Thus, future research should be focused on  
602 identifying biofilm phototrophic and heterotrophic species selected by toxicant exposures.  
603 Overall, by considering the development of co-tolerance or susceptibility to novel stressors,  
604 our study highlights the fact that the PICT concept may improve our ecological understanding  
605 of multiple metal contamination and specifically its consequences on diversity and ecosystem  
606 functioning.

607

## 608 **Acknowledgments**

609 The authors thank the Water Chemistry Laboratory of the CEMAGREF in Lyon who carried  
610 out nutrient analyses in water, M. Coquery, and J. Gahou for metal analysis in water and  
611 biofilms, B. Motte for field sampling and Peter Winterton for proofreading the English-  
612 language version of the manuscript. We thank the ONEMA (French National Agency for  
613 Water and Aquatic Ecosystems) for providing financial support.

614

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786 Table.1 Mean values ( $\pm$  standard deviation,  $n = 3$ ) of selected chemical parameters at the  
 787 Saint Joseph site (reference), within the control channels and within channels contaminated  
 788 by Cu, Zn and As, just before replacing water in the channels with water from the saint  
 789 Joseph site at the sampling date (i.e. week 5).

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Channel	pH	Oxy (mg.L <sup>-1</sup> )	cond. ( $\mu$ S.cm <sup>-1</sup> )	DOC (mg.L <sup>-1</sup> )	PO <sub>4</sub> <sup>3-</sup> (mg.L <sup>-1</sup> )	SiO <sub>2</sub> (mg.L <sup>-1</sup> )
<b>Saint-Joseph</b>	7.62 $\pm$ 0.04	8.94 $\pm$ 0.14	227.44 $\pm$ 1.54	2.63 $\pm$ 0.12	0.06 $\pm$ 0.01	14.67 $\pm$ 0.58
<b>Control</b>	7.37 $\pm$ 0.09	8.74 $\pm$ 0.28	278.00 $\pm$ 4.36	6.28 $\pm$ 0.52	0.04 $\pm$ 0.04	9.30 $\pm$ 2.20
<b>Cu</b>	7.49 $\pm$ 0.13	8.70 $\pm$ 0.12	278.00 $\pm$ 6.24	5.65 $\pm$ 0.44	0.02 $\pm$ 0.02	9.83 $\pm$ 2.40
<b>Zn</b>	7.55 $\pm$ 0.07	8.72 $\pm$ 0.40	287.00 $\pm$ 0.40	6.97 $\pm$ 0.32	0.03 $\pm$ 0.02	6.93 $\pm$ 0.76
<b>As</b>	7.55 $\pm$ 0.05	8.98 $\pm$ 0.08	295.00 $\pm$ 3.61	6.15 $\pm$ 0.22	0.03 $\pm$ 0.00	9.50 $\pm$ 3.82

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804 Table.2 Concentrations in  $\mu\text{g.L}^{-1}$  total and percentage of internalization within biofilms of  
805 metals (Cu, Zn and As) from the control and contaminated channels. Values correspond to the  
806 mean ( $\pm$  standard deviation) of concentrations obtained at the biofilms sampling date (n = 3).

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Channel	Total accumulated metals			% of Internalization		
	Cu	Zn	As	Cu	Zn	As
<b>Control</b>	104.3 $\pm$ 10.8	680.1 $\pm$ 20.8	51.6 $\pm$ 5.3	50.6 $\pm$ 2.3	60.7 $\pm$ 0.3	101.8 $\pm$ 0.7
<b>Cu</b>	448.9 $\pm$ 66.2	626.0 $\pm$ 106.1	53.1 $\pm$ 12.2	37.9 $\pm$ 2.3	57.2 $\pm$ 0.7	101.8 $\pm$ 2.5
<b>Zn</b>	95.7 $\pm$ 14.9	1461.5 $\pm$ 136.3	53.5 $\pm$ 5.1	42.1 $\pm$ 0.4	51.7 $\pm$ 4.5	100.6 $\pm$ 7.6
<b>As</b>	99.2 $\pm$ 18.3	709.1 $\pm$ 140.7	153.0 $\pm$ 19.6	51.9 $\pm$ 0.5	57.7 $\pm$ 5.1	99.1 $\pm$ 1.8

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824 Table.3 Long-term impact (mean values  $\pm$  standard deviation,  $n = 3$ ) of Cu ( $10 \mu\text{g.L}^{-1}$ ), Zn ( $30$   
825  $\mu\text{g.L}^{-1}$ ) and As ( $15 \mu\text{g.L}^{-1}$ ) on total biomass (AFDW), chlorophyll-a (chl-*a*), photosynthetic  
826 efficiency (Yield) and substrate induced respiration (SIR) of control, Cu-, Zn-, and As-  
827 exposed biofilms.

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Channel	AFDW ( $\text{g.m}^{-2}$ )	Chl-a ( $\mu\text{g.cm}^{-2}$ )	Yield	SIR
Control	10.33 $\pm 1.02$	8.06 $\pm 1.4$	0.42 $\pm 0.02$	135.03 $\pm 3.88$
Cu	11.74 $\pm 0.73$	8.2 $\pm 2$	0.47 $\pm 0.04$	118.09 $\pm 5.04$
Zn	9.41 $\pm 2.20$	8.5 $\pm 1.4$	0.45 $\pm 0.03$	115.56 $\pm 3.43$
As	9.63 $\pm 3.21$	1.8 $\pm 2.9$	0.38 $\pm 0.01$	114.25 $\pm 3.34$

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849 Table.4 Mean tolerance ( $\pm$  standard deviation,  $n = 3$ ) and tolerance or sensitivity ratios (R) (R  
 850  $> 1 =$  induced tolerance and  $R < 1 =$  induced sensitivity) determined for phototrophic (based  
 851 on photosynthesis bioassays) and heterotrophic (based on SIR bioassays) biofilm  
 852 communities sampled from the control channels and from contaminated channels with Cu, Zn  
 853 and As. Ratios were calculated by dividing the  $EC_{50}$  (pre-exposure) by the corresponding  
 854  $EC_{50}$  (control).

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	Control	Pre-exposure Cu	Pre-exposure Zn	Pre-exposure As
<b><math>EC_{50}</math> Photosynthesis bioassays (<math>\mu\text{M}</math>)</b>				
<b>Cu</b>	$37.6 \pm 5.1$	$99.5 \pm 24.8$	$77.3 \pm 7.4$	$108.2 \pm 9.3$
R	1	2.65	2.06	2.88
<b>Zn</b>	$1085.5 \pm 158.4$	$3216.5 \pm 650.1$	$2862.8 \pm 333.1$	$1094.6 \pm 114.9$
R	1	2.96	2.64	1
<b>As</b>	$14.0 \pm 2.1$	$3.4 \pm 0.8$	$7.2 \pm 1.1$	$3158.4 \pm 399.1$
R	1	0.24	0.51	224.51
<b><math>EC_{50}</math> SIR bioassays (<math>\mu\text{M}</math>)</b>				
<b>Cu</b>	$92.5 \pm 18.1$	$196.0 \pm 58.6$	$206.1 \pm 62.8$	$69.3 \pm 23.6$
R	1	2.12	2.23	1
<b>Zn</b>	$232.8 \pm 34.4$	$674.8 \pm 61.0$	$2596.3 \pm 80.0$	$275.4 \pm 39.3$
R	1	2.90	11.15	1
<b>As</b>	$1042.7 \pm 124.3$	$245.7 \pm 50.4$	$637.7 \pm 35.4$	$3586.1 \pm 130.0$
R	1	0.24	0.61	3.44

874 Table.5 Means ( $\pm$  standard deviation,  $n = 3$ ) of Shannon indexes (H) of the eukaryotic (euk)  
875 and prokaryotic (prok) biofilm communities sampled from the control channels and from  
876 contaminated channels with Cu, Zn and As.

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<b>Channel</b>	<b>H. euk</b>	<b>H. prok</b>
<b>Control</b>	4.81 $\pm 0.04$	4.86 $\pm 0.04$
<b>Cu</b>	4.35 $\pm 0.02$	4.44 $\pm 0.01$
<b>Zn</b>	4.33 $\pm 0.02$	4.43 $\pm 0.02$
<b>As</b>	4.36 $\pm 0.02$	4.34 $\pm 0.02$

899 Fig.1 Relative percentages of diatoms (□), cyanobacteria (■) and green algae (■) (determined  
900 by HPLC) in control channels and channels exposed to Cu, Zn and As.

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902 Fig.2 Principal component analysis (PCA) based on the relative abundance of the  
903 phototrophic pigments in the collected biofilms from the various channels. Arrows represent  
904 the correlation between the PCA axes (PC1 and PC2) and the pigments detected.

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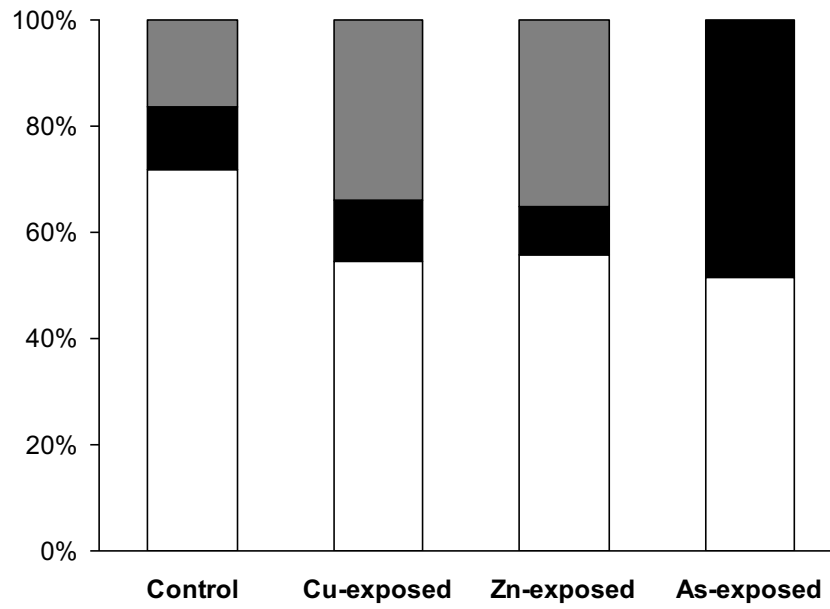
906 Fig.3 Principal component analysis (PCA) based on the relative band intensities obtained by  
907 PCR-DGGE analysis of the 18S rRNA gene fragment of the eukaryotic community in the  
908 biofilms collected from the various channels.

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910 Fig.4 Principal component analysis (PCA) based on the relative band intensities obtained by  
911 PCR-DGGE analysis of the 16S rRNA gene fragment of the prokaryotic community in the  
912 biofilms collected from the various channels.

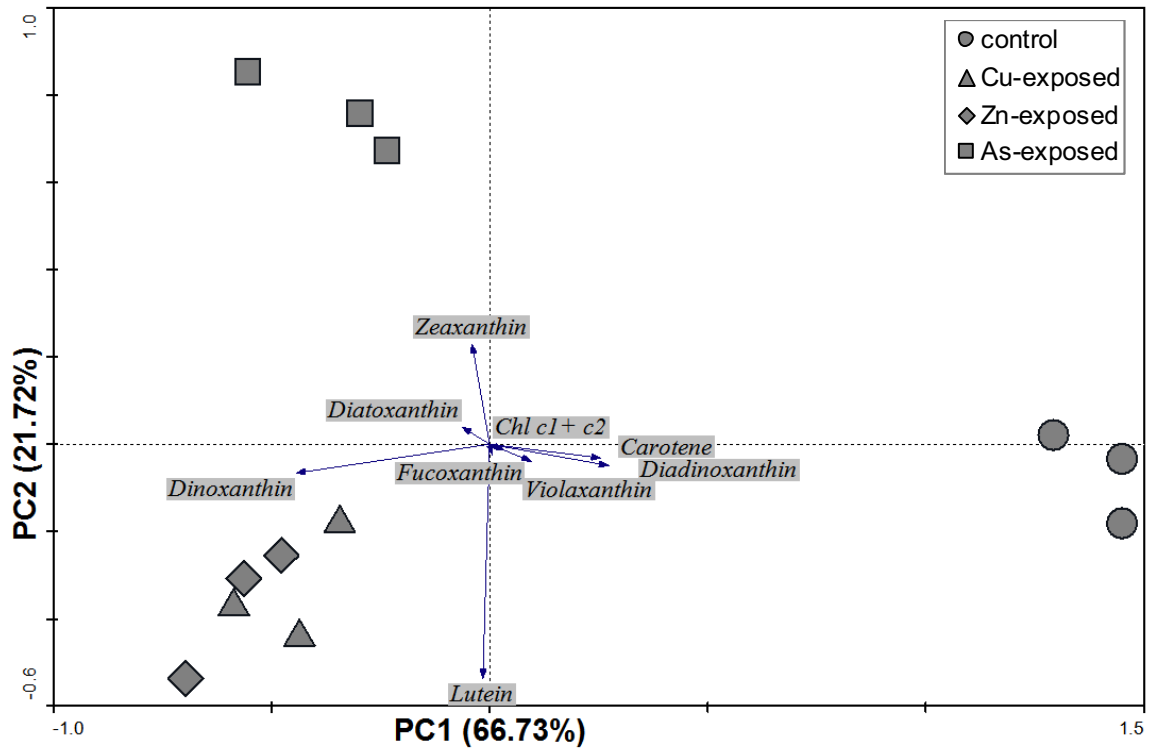
**Figure**

[Click here to download Figure: Fig1. relative abundance of phototrophs.ppt](#)



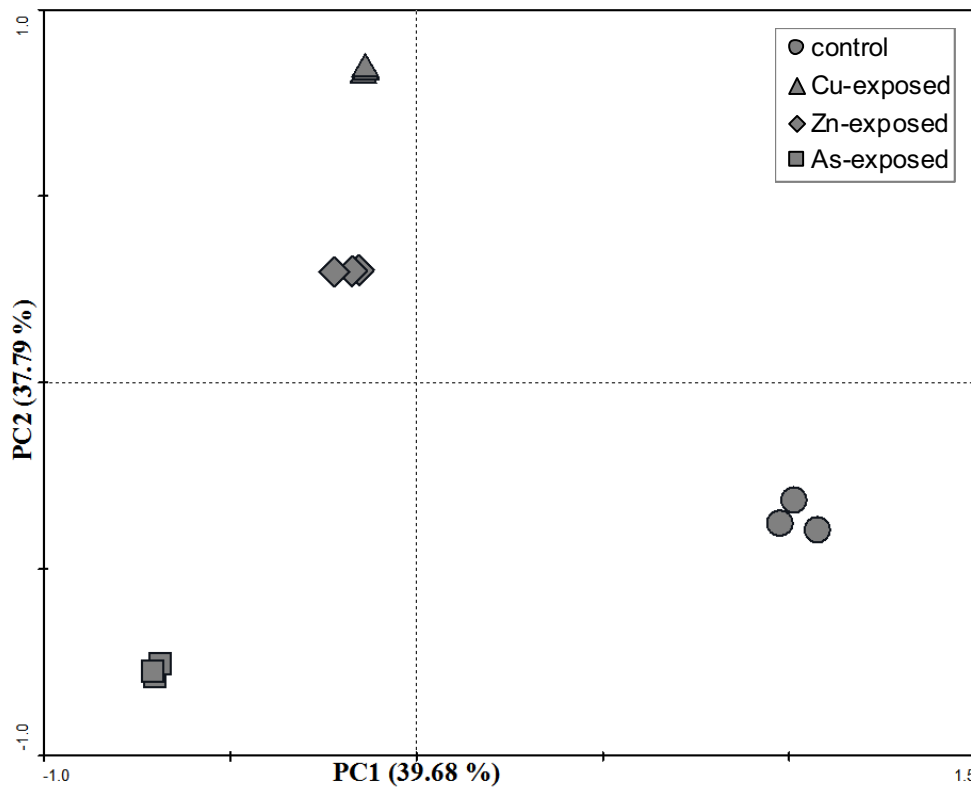
**Figure**

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**Figure**

[Click here to download Figure: Fig3. PCA eukaryotes.ppt](#)





**Figure**

[Click here to download Figure: Fig4. PCA prokaryotes.ppt](#)

