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

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Abstract

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

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

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1. Introduction

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

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

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

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

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

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

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

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

2. Material and methods

2.1. Sampling site

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

2.2. Experimental design

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

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

Biofilms colonized and grew for 5 weeks on artificial substrates (60 frosted glass disks of 1.5 cm^2 and 15 frosted glass slides of 18 cm^2) installed horizontally in each channel. At the start of the experiment, the three channel replicates were contaminated separately with one of the three tested metals with a nominal concentration of 10 $\mu g.L^{-1}$ of Cu, added as $CuSO_4$ (Merck, 99% purity), 35 $\mu g.L^{-1}$ of Zn added as $ZnSO_4$ (Sigma-Aldrich GmbH, 98% purity) and 15 $\mu g.L^{-1}$ of As added as Na_2HAsO_4 (Sigma-Aldrich GmbH; 98% purity). The three remaining channels were used as controls (no metals added). The toxicant (Cu, Zn and As) concentrations used in our study were similar to the maxima observed in Morcille river in late spring/early summer.

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

2.3. Biofilm sampling

After five weeks of growth, biofilms were sampled and analyses performed: Total biomass (glass disks), bioassays (photosynthetic efficiency and substrate-induced respiration 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), PO_4^{3-} and 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 µm, 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 cm^2) per channel were scraped using a polypropylene spatula and the
166 biofilm suspended in 40 mL of 0.2 µm Nuclepore-filtered water from the reference site. This
167 suspension was divided into two fractions. Fraction one (20 mL) was treated with 320 µL 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 µm
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

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

2.5. Total and phototrophic biomass

The organic matter content in 3 pooled small disks (4.5 cm² / channel) was calculated as described in Tlili et al. (2008). Results are expressed as $g.m^{-2}$. Chlorophyll-*a* content in biofilm was considered to be an indicator of the phototrophic biomass (Bonin and Travers, 1992) and was quantified by HPLC analysis. The chlorophyll-*a* was quantified using external calibrations of standard chlorophyll-*a* (C55H72MgN405, Carl Roth GmBH & Co). Final concentrations are given as $\mu g.cm^{-2}$ (see 2.7.1 for analytical details).

2.6. Biofilm function analysis

2.6.1. Photosynthetic efficiency

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

$$YII_{665nm} = \frac{F_m - F_0}{F_m}$$

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

2.6.2 Substrate-induced respiration

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

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_2 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_2-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 $CO_2.mg^{-1}AFDW.h^{-1}$. 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.

Stock solutions containing 2.10^{-1} M Cu ($CuSO_4$; Merck high-purity grade) or 2 M Zn ($ZnSO_4.7H_2O$; Sigma high purity grade) or 2.10^{-1} M As ($NaHAsO_4$; Sigma high-purity grade) were prepared in milli-Q water and stored at 4°C prior to dilution in the test vessels. Semi-logarithmic series of concentrations were freshly prepared by serial dilutions of the stock solutions in 0.2 µm-filtered water from the reference site. For photosynthetic bioassays (3 blanks and 3 replicates for each of the 6 increasing concentrations), final test concentrations ranged from 0 to 2.10^3 µM for Cu, from 0 to 6.10^3 µM for Zn and from 0 to 2.10^5 µM for As, whereas for SIR bioassays (3 blanks and 3 replicates for each of the 9 increasing concentrations) they ranged from 0 to 2.10^4 µM for Cu and As, and from 0 to 2.10^5 µM for Zn.

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

- *Short-term SIR bioassays*: Biofilm suspensions were distributed in deep wells (500 µL per well), to which we added 50 µL of the metal solutions (increasing concentrations). The microplate was then pre-incubated in the dark at room temperature for 3 hours (to cope with artefacts causing chemical release of CO_2), and then 30 µL of the glucose solution (120 $mg.mL^{-1}$) was added to each well and the detection microplate was positioned. Incubation and CO_2 measurements were processed afterwards as described above (section

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2.6.2). EC_{50} values were calculated from dose-response curves by plotting biofilm CO_2 production at each metal concentration.

2.7. Biofilm structure analysis

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

Three pooled glass disks (4.5 cm² / channel) were selected and processed for HPLC pigment analysis as described in Tlili et al. (2008). Identification of the different pigments was performed depending on their specific retention times and absorption spectrum using DAD according to SCOR (Jeffrey et *al*., 1997). A table was constructed (with samples as rows and pigments as columns) by taking into account the relative abundance of each pigment in a given sample (expressed as a percentage of the sum of the area of all the pigments in a sample). In addition, a quantitative method from a calculation model based on published ratios for cultures containing a mixture of different algae (Wilhem et al. 1991) was used to determine the relative abundance of diatoms, cyanobacteria and green algae from their specific pigment signatures (fucoxanthin, zeaxanthin and lutein, respectively). Final concentrations are given as $\mu g.cm^{-2}$.

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

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

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

2.8. Statistical data processing

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

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

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

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

2. Results

2.1. Physico-chemical data

Table 1

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

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

Table 2

Cu, Zn and As concentrations accumulated (total and % internalization) in biofilms are summarized in Table 2. The amounts of total Cu in the Cu-exposed biofilms were higher than those measured in the controls, and the same trends were observed for Zn and As 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**

2.4.1. Photosynthetic efficiency bioassay (Table. 4)

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

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

2.4.2. SIR bioassay (Table. 4)

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

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

2.5. Structural analysis

2.5.1. Phototrophic pigment analysis

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**

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

2.5.3. Prokaryotic diversity and richness

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

Fig. 4

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

4. Discussion

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The concentrations of dissolved nutrients, especially PO_4^{3-} and SiO_2 (phosphates are used by all organisms in biofilm, and silicates are principally used by diatoms) at the sampling dates in all the channels were very low compared to the initial values (reference site water), suggesting that a rapid uptake of nutrients had occurred in our experimental conditions. The DOC concentrations, however, increased in all the channels, suggesting autochthonous organic matter production by algae. Our physico-chemical measurements also showed a similar increase of the conductivity values in the contaminated channels but not in the control one. These results were expected since the addition of metal ions induces an increase in water conductivity (Guasch et al. 2002). Indeed, conductivity corresponds to the presence of anions and cations in water, and metal inputs in the water generally lead to the increase of these anions and cations (according to the metal speciation) and consequently to increased conductivity.

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

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

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

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

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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_2 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,

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

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

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

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

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

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

5. Conclusion

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

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

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786 Table.1 Mean values (± 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 ($mg.L^{-1}$)	cond. ($\mu S.cm^{-1}$)	DOC ($mg.L^{-1}$)	PO_4^{3-} ($mg.L^{-1}$)	SiO_2 ($mg.L^{-1}$)
Saint-Joseph	7.62 ± 0.04	8.94 ± 0.14	227.44 ± 1.54	2.63 ± 0.12	0.06 ± 0.01	14.67 ± 0.58
Control	7.37 ± 0.09	8.74 ± 0.28	278.00 ± 4.36	6.28 ± 0.52	0.04 ± 0.04	9.30 ± 2.20
Cu	7.49 ± 0.13	8.70 ± 0.12	278.00 ± 6.24	5.65 ± 0.44	0.02 ± 0.02	9.83 ± 2.40
Zn	7.55 ± 0.07	8.72 ± 0.40	287.00 ± 0.40	6.97 ± 0.32	0.03 ± 0.02	6.93 ± 0.76
As	7.55 ± 0.05	8.98 ± 0.08	295.00 ± 3.61	6.15 ± 0.22	0.03 ± 0.00	9.50 ± 3.82

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804 Table.2 Concentrations in $\mu g.L^{-1}$ 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 (± 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
Control	104.3 ± 10.8	680.1 ± 20.8	51.6 ± 5.3	50.6 ± 2.3	60.7 ± 0.3	101.8 ± 0.7
Cu	448.9 ± 66.2	626.0 ±106.1	53.1 ± 12.2	37.9 ± 2.3	57.2 ± 0.7	101.8 ± 2.5
Zn	95.7 ± 14.9	1461.5 ± 136.3	53.5 ± 5.1	42.1 ± 0.4	51.7 ± 4.5	100.6 ± 7.6
As	99.2 ± 18.3	709.1 ± 140.7	153.0 ± 19.6	51.9 ± 0.5	57.7 ± 5.1	99.1 ± 1.8

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Table.3 Long-term impact (mean values ± standard deviation, n = 3) of Cu (10 µg.L^{-1}), Zn (30 µg.L^{-1}) and As (15 µg.L^{-1}) on total biomass (AFDW), chlorophyll-a (chl-*a*), photosynthetic efficiency (Yield) and substrate induced respiration (SIR) of control, Cu-, Zn-, and As-exposed biofilms.

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

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

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

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

Channel	H. euk	H. prok
Control	4.81 ± 0.04	4.86 ± 0.04
Cu	4.35 ± 0.02	4.44 ± 0.01
Zn	4.33 ± 0.02	4.43 ± 0.02
As	4.36 ± 0.02	4.34 ± 0.02

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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.
901
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.
905
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.
909
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.

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Figure

Click here to download Figure: Fig1. relative abundance of phototrophs.ppt

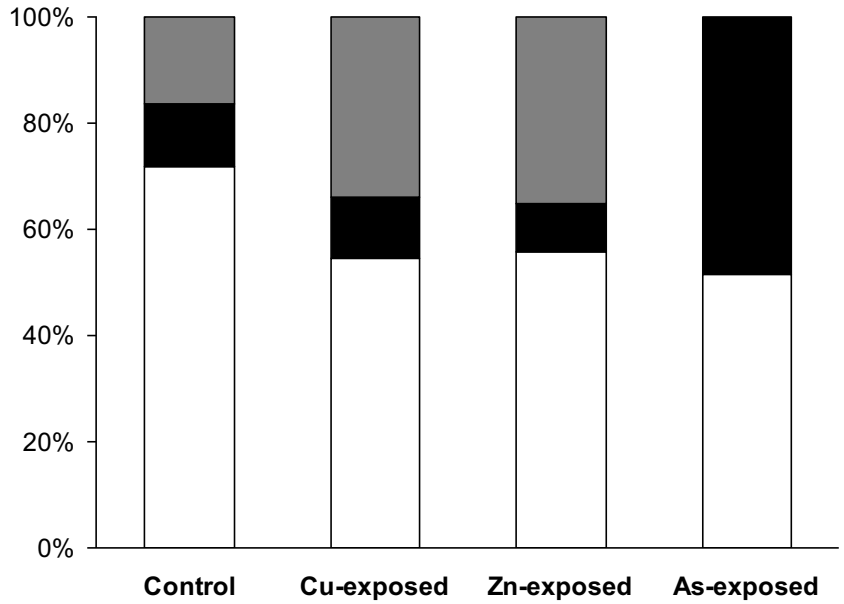

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Figure

Click here to download Figure: Fig2. PCA pigments.ppt

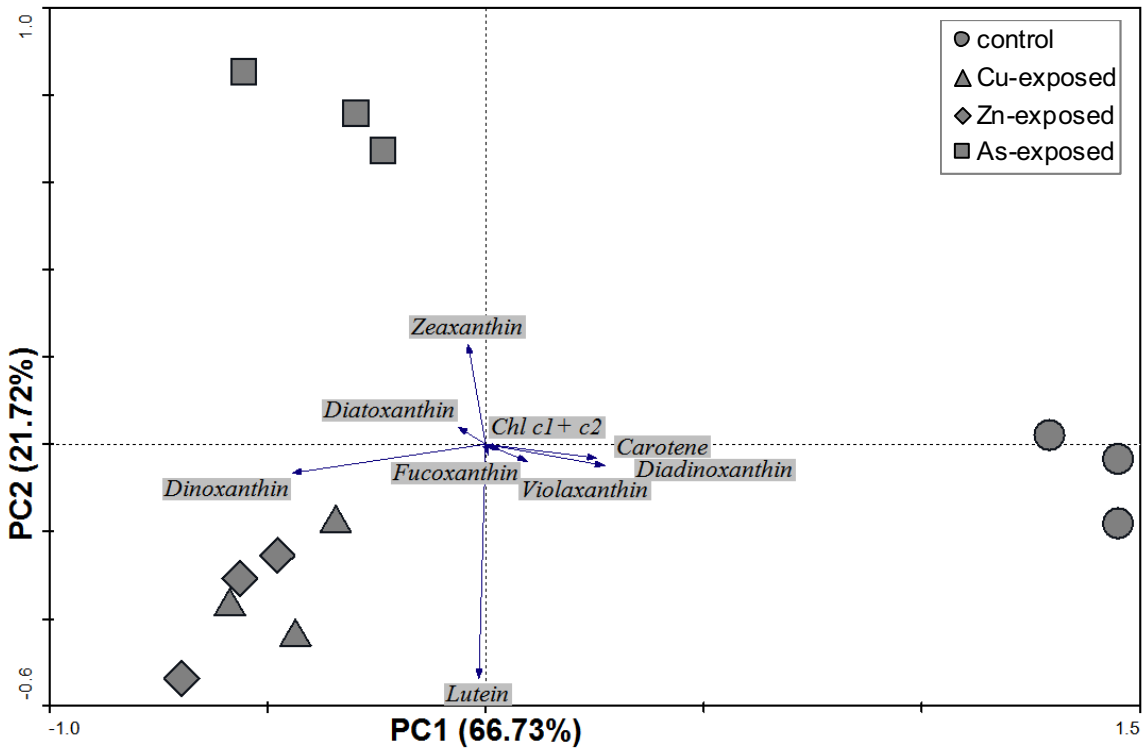

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Figure

Click here to download Figure: Fig3. PCA eukaryotes.ppt

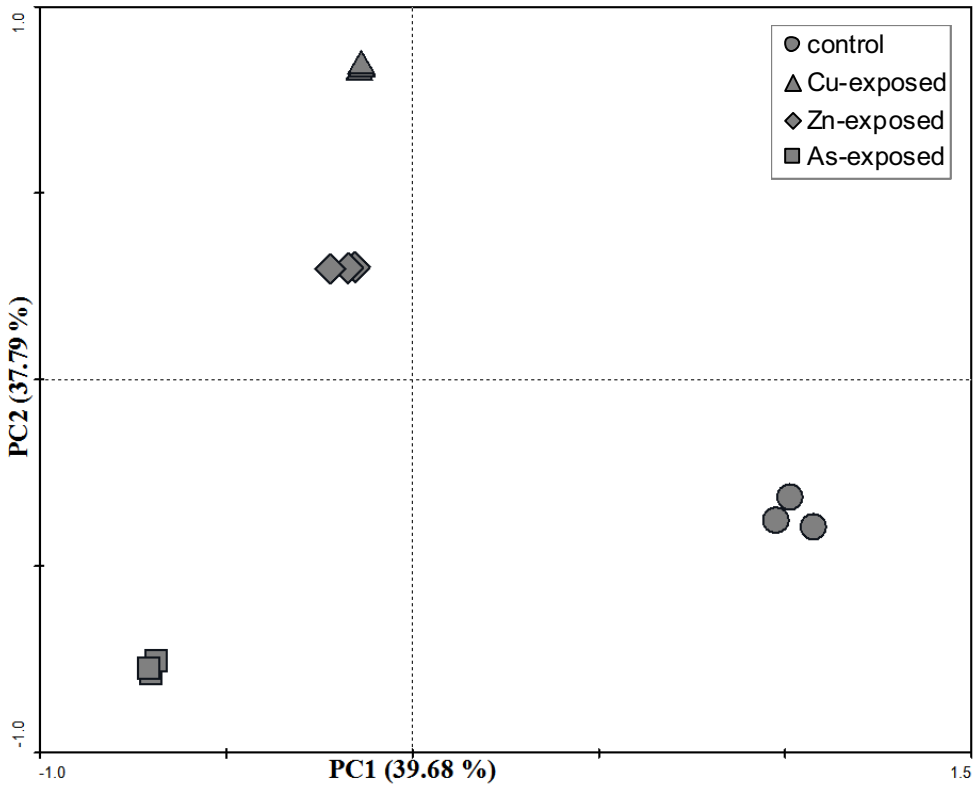

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Figure

Click here to download Figure: Fig4. PCA prokaryotes.ppt