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Linking community tolerance and structure with low metallic contamination: A field study on 13 biofilms sampled across the Seine river basin

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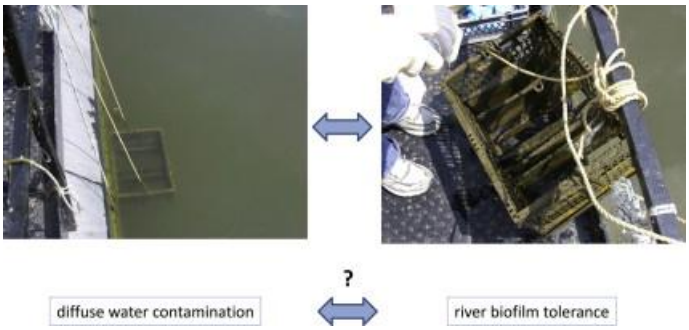
Abstract:

It is difficult to assess the biological consequences of diffuse water contamination by micropollutants which are present in rivers at low, even sublethal levels. River biofilms, which respond quickly to changes of environmental parameters, are good candidates to acquire knowledge on the response of aquatic organisms to diffuse chemical contamination in the field. The study was designed as an attempt to link biofilm metal tolerance and metallic contamination in a field survey covering 13 different sampling sites in the Seine river basin (north of France) with low contamination levels. Cd and Zn tolerance of heterotrophic communities was assessed using a short-term toxicity test based on β-glucosidase activity. Metal tolerance levels varied between sites but there was no obvious correlation between tolerance and corresponding water contamination levels for Cd and Zn. Indeed, metallic contamination at the sampling sites remained subtle when compared to water quality standards (only two sampling sites had either Zn or both Cu and Zn concentrations exceeding the Environmental Quality Standards set by the EU Water Framework Directive). Yet, multivariate analysis of the data using Partial Least Squares Regression revealed that both metallic and environmental parameters were important variables explaining the variability of metal tolerance levels. Automated Ribosomal Intergenic Spacer Analysis (ARISA) was also performed on both bacterial and eukaryotic biofilm communities from the 13 sampling sites. Multivariate analysis of ARISA fingerprints revealed that biofilms with similar tolerance levels have similar ARISA profiles. Those results confirm that river biofilms are potential indicators of low, diffuse contamination levels of aquatic systems.

Graphical abstract :



Highlights

► River biofilms were collected on 13 sites with low metallic contamination levels. ► Multivariate analysis shows that biofilm metal tolerance was linked to contamination. ► Environmental parameters also modulate biofilm tolerance levels. ► Biofilms with similar tolerance levels have similar ARISA profiles. ► Biofilms are potential sensitive indicators of diffuse, low-level contamination.

Keywords: River biofilm ; Metal tolerance ; Toxicity ; ARISA ; PICT ; Community-level response

1. Introduction

Over the last decades, several authors have discussed the importance of measuring biological responses at the community level in ecotoxicological studies (Clements and Rohr 2009, Geiszinger et al. 2009). Indeed, current risk-assessment of toxicants remains largely based on standardized, single-species tests performed in the laboratory, the results of which are then extrapolated at the ecosystem level. Yet, the responses of single-species tests might differ from responses occurring at the community level as important aspects of community ecology are not considered: for instance, interactions between species within the exposed communities are not taken into account (Schmitt-Jansen et al. 2008). Some studies have even suggested that current environmental quality standards might thus not always be sufficient to protect organisms at the community level (McClellan et al. 2008). Moreover, it is generally acknowledged that community endpoints such as species richness and diversity are intimately linked to ecosystem responses to stress and therefore essential to maintain ecosystem services (Clements and Rohr 2009).

A community-level approach like PICT (Pollution-Induced Community Tolerance) is an interesting ecotoxicological tool to assess the impacts of toxicant exposures (Blanck et al. 2003). The PICT approach proposes to assess shifts in community composition (from a sensitive community to a more tolerant one) due to toxic exposures. It relies on the assumption that sensitive components from the original community (species, genotypes or phenotypes) will be gradually replaced by more tolerant ones during exposure, thus leading to an increase of the global community tolerance. Tolerance development is measured as a shift in the Effect Concentration (usually EC_{50}) or Lethal Concentration (LC_{50}) obtained with a short-term toxicity

test based on a physiological endpoint. Tests can be conducted on communities grown in artificial environments (microcosms, mesocosms) or directly on communities collected *in situ*. Interpretation of PICT measurements has proved to be more difficult in field studies either because of co-tolerance, which occurs for chemicals with similar modes of actions or through the development of unspecific defense mechanisms (such as mucilage for algal and bacterial communities) (Schmitt-Jansen et al. 2008, Soldo and Behra 2000) or because of environmental factors, such as light, nutrients, etc. (Serra et al. 2010, Guasch et al. 2002), which also affect tolerance levels.

However, several PICT studies have investigated river biofilm tolerance to metals or herbicides in the field and succeeded in linking tolerance acquisition to toxic exposure mostly by focusing on one chemical (for instance zinc or atrazine: Blanck et al. 2003, Tlili et al. 2011, Pesce et al. 2010, Admiraal et al. 1999) or more recently on multi-metallic pollution (Fechner et al. 2012a, Fechner et al. 2012b), and usually in the same river upstream to downstream from a polluted area. However, field studies attempting to link biological effects and chemical contamination over wide ranges of sampling sites remain scarce. Moreover, current contamination levels are characterized by large numbers of chemicals at low exposure concentrations, which means that for historic contaminants like metals, the issue has shifted from managing acute effects of single toxicants at high exposure levels (which might still occur for instance in areas impacted by mining activities in the case of metals) to managing more subtle, chronic effects of mixtures of chemicals (Schmitt-Jansen et al. 2008). Recent studies, including the PICT studies mentioned above, point out that microbial communities might be good indicators of chemical contamination, even of complex mixtures of chemicals at low exposure levels such as found in the field. Indeed, microbial communities, which undergo fast changes in

composition and function in response to changes in environmental parameters, are acknowledged as potential interesting bioindicators of contamination (Sims et al. 2013, Sun et al. 2012, Ricciardi et al. 2009). For instance, Sun et al. (2012) have succeeded in linking variations in microbial community composition in sediment and environmental parameters including sediment metallic contamination using Automated-Ribosomal Intergenic Spacer Analysis (ARISA) data collected over six estuaries sites in Australia. In another recent study, Ancion et al. (2013) also managed to link biofilm-associated metals with variations of bacterial communities using ARISA fingerprints. However, to our knowledge, there are no field studies using tolerance measurements on biofilms from a wide range of sampling sites impacted only by low, diffuse multi-metallic pollution.

The present study was designed as a first attempt at linking metal tolerance and bacterial and eukaryotic community composition of river biofilms to environmental parameters (including physico-chemical parameters and metallic contamination levels) over a wide range of sampling sites in the Seine river basin. Sampling sites were chosen to give a broad representation of contamination levels in a large area impacted by diffuse, low metallic contamination (compared to environmental quality standards). Biofilms were collected at 13 sites in the Seine river basin (North of France) and their Cd and Zn tolerance levels were measured using a short-term toxicity test based on β-glucosidase activity (which measures the tolerance of heterotrophic communities). In parallel, bacterial and eukaryotic community composition was investigated using ARISA. This fingerprinting technique, which exploits the length polymorphism of the 16S-23S intergenic spacer of bacteria and the ITS1-5.8S-ITS2 region of eukaryotes, had already proved useful to assess shifts in community composition upstream to downstream from Paris in the Seine river in previous studies (Fechner et al. 2012a, 2012b). The present study provides a

109 larger survey of river biofilms and their use as possible indicators of urban contamination in a
110 context of diffuse, low and multi-metallic exposure.
111

2. Materials and Methods

2.1 Collection of river biofilms

River biofilms were collected at 13 sites located in the Seine river basin (North of France, Figure 1) after a two-weeks colonization period (see below for details about the collection of biofilm samples).

Sites were sampled over three times from summer (sites 1 to 4 were sampled in early September 2009) to autumn of the same year (sites 5 to 9 in October 2009 and sites 10 to 13 in November 2009). Biofilms were collected on Low Density PolyEthylene membranes (4 to 7 membranes of 14 * 25 cm^2) vertically attached to plastic crates, as described elsewhere (Fechner et al. 2010a), immersed at each sampling site for two weeks at the edge of the river. Colonized membranes were carried back to the laboratory in 250 mL glass-bottles placed in a cooler and filled with mineral water (Grand Barbier, Mont-Dore, France, see Table S3). They were then hand-scraped to remove periphyton and make biofilm suspensions in mineral water as in Fechner et al. (2010a). Biofilms scraped from the membranes collected at one site were pooled together to provide a unique biofilm suspension for each date and each site. Aliquots of the biofilm suspensions were then assigned to various analyses for periphyton characterization or tolerance measurements. To obtain ARISA fingerprints, 50 mL aliquots of biofilm suspensions (three aliquots ie pseudo-replicates per sampling site) were centrifuged (15 minutes, 10 000 g, 4°C). Supernatants were discarded and pellets were stored at -80°C for further use.

2.2 Water chemistry and metallic contamination

Total and dissolved metal concentrations (Cd, Cu, Ni, Pb, Zn, Co, Cr, Mn) as well as 17 physico-chemical parameters (temperature, pH, conductivity, Total Suspended Solids

concentration, Dissolved and Particulate Organic Carbon – DOC and POC – in water, as well as major ions: HCO_3^-, Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-}, $N0_3^-$, PO_4^{3-}, NO_2^-, NH_4^+, see Table S1 for detailed values) were measured at week 0, 1 and 2 of the study at each site. Labile metal concentrations were measured using Diffusive Gradients in Thin films (DGTs: Zhang and Davison 1995) immersed in the river at each site for one and two weeks (three replicates each).

To describe the metallic contamination of each sampling site in a more integrated way, we also used the cumulative criterion unit (CCU) described by Clements et al. (2000) as the ratio of the measured metal concentration (dissolved metal concentrations were used) to the US EPA national recommended water quality criterion (National Recommended Water Quality Criteria 2009), summed for Cd, Cu, Ni, Pb, Zn and Mn at each sampling site:

$$\mathrm{CCU} = \sum m_i / c_i$$

where m_i is the total dissolved metal concentration and c_i the criterion value for the ith metal. Criteria were corrected to account for modifications of water hardness for Cd, Cu, Ni, Pb and Zn and a chronic criterion of 100 µg/L was used for Mn according to US-EPA guidelines (National Recommended Water Quality Criteria 2009, see Table S2). In this study, we used CCU to provide a unique, global value to compare sampling sites based on dissolved metal contamination levels and general metal toxicity which is useful to assess statistical correlations between biological responses (biofilm tolerance acquisition and structure modification) and contamination data.

157 ***2.3 Biofilm characterization: biofilm descriptors and ARISA fingerprints***

158 Biofilms' dry weight (DW), ash-free dry weight (AFDW), and chlorophyll a (Chl-a)
159 concentrations were measured as described elsewhere (Fechner et al. 2010b). ARISA
160 fingerprints were obtained at each sampling date as in Fechner et al. (2010b). Briefly, DNA was
161 extracted from biofilm pellets using the Power Soil DNA Isolation Kit (Mobio Laboratories, Inc.,
162 Carlsbad, US). PCR amplification of bacterial 16S-23S ITS was performed using primers
163 ITSF/ITSReub with 1 X PCR buffer, 1.25U of Thermo-Start Taq DNA polymerase (ABGene
164 Ltd, Epsom, UK), 0.2mM (each) dNTP, 1.5mM of $MgCl_2$, 0.5 mg/mL bovine serum albumin and
165 0.5 mM ITSF and ITSReub (each). The mixture was held at 94 °C for 3 min, followed by 35
166 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min and a final extension step at 72 °C
167 for 10 min. Amplification of eukaryotic ITS1-5.8S-ITS2 regions was performed using primers
168 2234C/3126T and a similar protocol (annealing temperature: 57.5 °C). Amplicons were
169 separated on an electrophoresis Bioanalyzer (2100 Electrophoresis Bioanalyzer, Agilent
170 Technologies) and fluorescence data was converted into electrophoregrams using 2100 Expert
171 software (Agilent Technologies). Electrophoregrams of pseudo-replicates were averaged in order
172 to obtain one ARISA profile per sampling site. ARISA profiles were then processed using the
173 StatFingerprints R package (Michelland et al., 2009).

174

175 ***2.4 Metal tolerance measurements***

176 Cd and Zn tolerance levels of heterotrophic communities from sampled biofilms were
177 assessed using the normalized EC_{50} values obtained with the toxicity test based on β-glucosidase
178 activity developed by Fechner et al. (2010a, 2011). Briefly, biofilms were exposed to metals for
179 one hour (at least six concentrations of metal varying between 0.001 and 10 mM were tested in

triplicate for each toxicity test). Metal exposure levels during the toxicity tests were checked by measuring metal concentrations in the stock solutions by flame AAS (Varian Inc., USA). β-glucosidase activity of the metal-exposed biofilms was measured spectrofluorometrically using Methylumbelliferyl-β-D-glucopyranoside (Sigma-Aldrich). Fluorescence of 4-Methyllumbelliferone (MUF) was measured using a LB 941 Tristar Ti fluorescence microplate reader (Berthold Technologies, Bad Wildbad, Germany). Fluorescence measurements were converted into MUF concentrations by calibrating the spectrofluorimeter with a range of MUF solutions in demineralised water. Dose/effect curves were obtained by plotting the % inhibition of β-glucosidase activity as a function of metal concentration, and by fitting the data to the Hill model and adjusting each parameter (maximum % of inhibition, EC_{50} which is the concentration at which 50% of the enzymatic activity is inhibited and Hill number) by non-linear regression. 95% confidence intervals around the fitted parameters were estimated using a Bootstrap method. Community tolerance to metal was then assessed by calculation of a normalized EC_{50} value by dividing the EC_{50} value by the Total Suspended Solids (TSS) concentration of the biofilm suspension used for the short-term test (Fechner et al. 2010a).

This normalized EC_{50} value has been shown to provide a reliable and robust estimation of metal tolerance that does not depend on experimental conditions (namely, the quantity of biofilm used for the test), both on field and laboratory-exposed biofilms (Fechner et al. 2012a, 2010a, 2011).

2.5 Multivariate analysis

PCA was performed on all the environmental parameters measured at the 13 sampling sites including metallic contamination levels (mean values of physico-chemical data and metal concentrations were used).

The effects of metal contamination on river biofilms' tolerance levels were examined using Partial Least Squares (PLS) regression. PLS is a regression extension of PCA which is used to connect two blocks of variables (X and Y) to each other. It derives its usefulness from its ability to analyze data with noisy, collinear or even incomplete variables. Moreover, the precision of the PLS model increases with the number of X variables (Eriksson et al. 2006). In the present study, PLS regression was performed with Cd and Zn tolerance levels and % maximum of inhibition (obtained during short-term tests performed with Cd and Zn) as the Y variables and the environmental parameters of the river water (physico-chemical parameters, metal concentrations) and biofilm descriptors (DW, AFDW and Chl-a) as the X-variables. The quality of a PLS regression model can be assessed both in terms of goodness of fit (the explained variance of both X and Y-variables calculated as R^2X and R^2Y) and goodness of prediction (Q^2) which is evaluated by cross-validation. The importance of each X-variable in the regression model is summarized by its Variable Influence in the Projection (VIP) values, which takes into account the amount of explained Y-variance and may be used to discriminate between X-variables. PLS regression was performed using XLStat 2010 (Addinsoft, France). Before the PLS regression, all data was log-transformed and normalized.

The influence of the environmental parameters on ARISA fingerprints was investigated using non metric Multidimensional scaling (nMDS) performed on the Euclidian distances between profiles (n = 1000 permutations). nMDS constructs a three-dimensional map showing each

fingerprint as one plot so that highly similar fingerprints are plotted together. A stress value is used to compute a goodness of fit analysis between the reproduced (in the nMDS map) and the actual distances. A stress value <0.1 corresponds to a good ordination (Clarke 1993).

Analysis of similarity (ANOSIM) was also performed on the Euclidian distance matrix of both bacterial and eukaryotic profiles (1000 permutations) to test the effect of grouping fingerprints according to tolerance and CCU (for each parameter, profiles were grouped together as “high” or “low” whether the corresponding parameter was above or below the mean calculated for all 13 sites). ANOSIM is a non-parametric permutation procedure which tests whether differences in dissimilarity between groups exceed differences within groups. It generates a global test statistic (R) which varies between −1 and 1 and the magnitude of which indicates the degree of separation between groups of samples. A value of 1 indicates complete separation whereas a value of 0 indicates no separation.

3. Results and discussion

3.1 Linking biofilm tolerance and metal contamination

The PCA score plot (Fig. 2a) separates sampling sites with higher contamination levels (CCU > 0.30 for sites 4, 7, 8, 10, 11 and 13, Table 1) from the other sites along the first principal component which is correlated to metallic contamination (Fig. 2b). Distribution of sampling sites is less relevant along the second component (only 13.5% of variability) but groups sites with high HCO_3^- and pH levels on the upper part of the graph (sites 7, 8, 9, 12, 13). Metal concentrations (Table 2 and Table S4) remained low at all sampling sites for instance when compared to environmental quality standards (EQS) of the Water Framework Directive (WFD) or national recommended water quality criteria from the US-EPA. The only concentrations that

246 exceeded quality standards (in this case WFD EQS) were for Cu and Zn at site 10 (with
247 dissolved concentrations of 1.9 and 9.1 µg/L respectively) and for Zn at site 13 (with a dissolved
248 Zn concentration of 46.7 µg/L, which is considerably higher than the other sampling sites, the
249 mean dissolved Zn concentration being 6.5 µg/L, Table 2). Dissolved Cu also reached the WFD
250 EQS (1.4 µg/L) at site 4. In contrast, other PICT studies performed on field-sampled periphyton
251 report higher contamination levels, often due to former mining activities (Blanck et al. 2003,
252 Tlili et al. 2011, Admiraal et al. 1999, Lehmann et al. 1999). For instance, total Zn
253 concentrations reported in a large field study linking biofilm PICT measurements and Zn
254 exposure concentrations (Blanck et al. 2003) over 15 sites across Europe varied between 3.9
255 µg/L and more than 2 mg/L with a mean of 195.2 µg/L (in the present study the mean total Zn
256 concentration is 13.6 µg/L and, except for a high concentration of 60.0 µg/L at site 13, total Zn
257 concentrations vary from 2.7 to 26.1 µg/L, Table S4). Similarly, the mean dissolved Zn
258 concentration at the reference site in another study (Tlili et al. 2011) is 36 µg/L and reaches 454
259 µg/L at the most contaminated sampling site. The 13 sites of the present study therefore reflect
260 current urban contamination in the Seine river basin which is characterized by a mixture of
261 micropollutants at particularly low, sublethal concentrations (Uher et al. 2011, Tusseau-
262 Vuillemin et al. 2007). Field studies linking biological responses, especially community-level
263 parameters, and such low metallic contamination levels are scarce.

264 Metal tolerance levels (Table 1) varied between sites (5.9 and 8.0 factors were found between
265 the lowest and highest tolerance levels for Cd and Zn respectively). No correlation was found
266 between tolerance and corresponding contamination levels for Cd and Zn (for instance Pearson
267 correlation coefficient R = 0.08 and 0.13 for dissolved Cd/Cd tolerance and dissolved Zn/Zn
268 tolerance respectively) or between tolerance and CCU (R = 0.12 and 0.22 for Cd and Zn

respectively). The absence of correlation is not totally surprising considering the very low metallic concentrations registered at those sampling sites. The maximum % inhibition of β-glucosidase activity were estimated along with EC_{50} values from the dose/effect curves by fitting the test data to the model (Table 1). A maximum % inhibition below 100% means that β-glucosidase activity cannot be fully inhibited during short-term metal exposure even at high metal concentrations. A decrease of this parameter has previously been observed as tolerance levels increased for biofilms exposed to metals either in the laboratory or in the field (Fechner et al. 2012a, 2011, 2012c). In the present study, the maximum % inhibition is also negatively correlated to tolerance ($R = 0.72$, $p - value = 0.006$ for Cd and $R = 0.49$, $p - value = 0.09$ for Zn) and even to CCU ($R = 0.61$, $p - value = 0.03$ for Cd and $R = 0.62$, $p - value = 0.02$ for Zn). These results confirm that variations in maximum % inhibition provide an additional biological response of river biofilms to toxic exposure.

Those results also confirm that, as could be expected for a field study covering a rather large geographical area and different sampling sites with low contamination levels, establishing a link between tolerance and contamination is not obvious. For instance, the biofilm collected at site 1 has the highest Cd and Zn tolerance levels (Table 1) whereas metal concentrations at this site were all below the mean calculated for the 13 sites (except for total Cu see Table S4 for details). This sampling site is also characterized by a low conductivity, TSS, pH and low ionic concentrations (see Table S1 for details). It is thus possible that physico-chemical characteristics of the river water play a role on metal speciation and bioavailability *in situ* and consequently on biofilms' response to metals. Environmental parameters other than metallic concentrations must definitely be taken into account to correctly interpret tolerance measurements, especially in the case of low exposure levels which do not engender strong and obvious tolerance development.

292

293 *3.2 Community tolerance: an integrative response to environmental parameters?*

294 Multivariate analysis was thus used to explore further the relationships between metal
295 tolerance, maximum % inhibition and all the environmental parameters likely to modulate
296 community tolerance. PLS produced a six-component regression model with a good fit (84 and
297 97% of explained variance for the X and Y variables respectively) and with good predictive
298 ability ($Q^2 = 0.78$, see Table S5 for details on model parameters). PLS revealed that both metallic
299 and physico-chemical parameters were important variables explaining the variability of metal
300 tolerance levels (Table 3).

301 Among the most influential parameters in the regression model (VIP > 1), Zn and Cd
302 concentrations (total for both metals and also dissolved Zn) appear, as well as other metallic
303 concentrations (Cr, Pb and Ni). Finding metallic concentrations other than Cd and Zn among the
304 influential variables is not totally surprising as co-tolerance phenomenons can be due to either
305 specific or non-specific defense mechanisms. Among specific defense mechanisms, evidence of
306 multi-resistance to Pb, Cd and Zn (a family of transport protein) has already been described in
307 bacterial species (Nies 2003). Multi-resistance to Co, Zn, Cd and Ni has equally been linked to
308 the presence of export proteins (Nies 2003) which is interesting as Co also appears as one of the
309 important X variables in the regression model (VIP > 0.95).

310 It is also interesting to note that for Cd and Zn, labile concentrations have a lower significance
311 in the regression model (VIP < 0.9) than total concentrations. DGT-labile metal concentrations
312 are an estimate of time-weighted average concentrations of inorganic and weakly-complexed
313 dissolved metals (Tusseau-Vuillemin et al. 2007). In a previous study on three sites located along
314 the Seine river (Fechner et al. 2012a), multivariate analysis suggested that labile and dissolved

315 metal concentrations might be better related to tolerance acquisition for metals like Cd and Zn
316 than total metal concentrations. Indeed DGT-labile metals have been shown to provide
317 reasonably good estimations of bioavailable metal concentrations to various aquatic organisms
318 compared to total and dissolved metals (Buzier et al. 2006). Yet labile copper concentrations
319 have also been shown to underestimate copper bioavailability to bryophytes, especially at rather
320 low concentrations (Ferreira et al. 2008). As regards biofilms more specifically, metal
321 bioavailability and toxicity mechanisms towards biofilm microorganisms are complex and
322 depend on the biofilm sample, the metal considered and metal speciation (Bradac et al. 2010,
323 Meylan et al. 2004). More field data needs to be obtained in order to investigate further the link
324 between metal speciation *in situ* and biofilm metal tolerance.

325 pH, POC, NH_4^+, Na^+, Mg^{2+} and water alkalinity are also found among the most important
326 physico-chemical parameters. The coefficients associated with the Y variables (Table 3) show
327 that an increase in pH, Mg^{2+}, Na^+ and alkalinity are all negatively correlated with tolerance
328 acquisition in the regression model. This is not totally surprising as a parameter like Mg^{2+} is
329 known to influence metal bioavailability by inhibiting the complexation of metal ions to
330 biological ligands and thus might decrease metal toxicity towards aquatic organisms (Niyogi and
331 Wood 2004) (and thus probably tolerance acquisition by biofilm microorganisms). Similarly, an
332 increase of pH or water alkalinity might be correlated with a decrease of tolerance as both
333 influence metal speciation (for instance by metal precipitation or by the appearance of other toxic
334 inorganic species such as $MeOH^+$) and therefore metal toxicity (Niyogi and Wood 2004). The
335 influence of parameters like POC and NH_4^+ is less easy to interpret. POC might be a source of
336 contamination for biofilm microorganisms as metal-contaminated particulate matter from the
337 river water might bind to the biofilm matrix. Moreover, Na^+, POC, NH_4^+ or any environmental

parameter might play a role in the species composition of the sampled biofilms and thus in their sensitivity to metals: pH could be a good example as it has a strong influence on bacterial community structure (Fierer et al. 2007). It is also interesting to note that biofilm descriptors such as biofilm DW and Chl-a are among the most influential variables as those parameters were not correlated with tolerance levels in a previous study on biofilms collected along the Seine river (Fechner et al. 2012a). Modifications of those biofilm descriptors might also indicate changes in biofilm species composition (for instance a varying Chl-a concentration might reveal changes in algal species composition) or in the matrix composition (a change in DW might be related to a modification of exopolysaccharides - EPS - production in the matrix or an increase of mineral particles from the river being retained within the matrix). Analysis of ARISA fingerprints brings further information on the modifications of biofilm community structure among the 13 sampling sites.

3.3 Linking community composition and metal tolerance

Tolerance acquisition can be related to genetic and/or phenotypic adaptation and selection processes within the exposed community. In an attempt to better understand the genetic mechanisms at the root of tolerance acquisition in the 13 biofilms sampled in this study, we used ARISA as a fast and efficient technique to visualize gross genetic modifications of community structure. nMDS of both bacterial and eukaryotic ARISA fingerprints was used to visualize groups of biofilms with similar structure (assessed with ARISA fingerprints). Interestingly, bacterial profiles (Figure 3a shows a 2D projection of the 3D nMDS plot) appear to be grouped according to Zn tolerance levels as confirmed by ANOSIM (Global ANOSIM, $R = -0.175$, p-value = 0.08) and not Cd tolerance levels ($R = -0.021$, p-value = 0.49). Similarly, eukaryotic

profiles (Figure 3b) are better grouped according to Zn tolerance levels (R = 0.147, p-value = 0.1) than to Cd tolerance levels (R = 0.03, p-value = 0.33). Moreover, ANOSIM reveals that ARISA profiles are also grouped according to CCU levels for eukaryotic communities (R = 0.197, p-value = 0.14) and to a lesser extent for bacterial communities (R = -0.206, p-value = 0.92). nMDS reveals here that differences/similarities in community structure of both bacterial and eukaryotic microorganisms are related to differences/similarities between tolerance levels. Therefore it is probable that selection processes (disappearance of sensitive species and development of metal-tolerant ones) were involved in tolerance acquisition. It is also probable that metal contamination (characterized here by CCU levels) modifies community structure. However, it is difficult to interpret further modifications of ARISA fingerprints and their link to environmental parameters or tolerance levels.

Although ARISA remains a technique for a global, gross analysis of community structure (only main Operational Taxonomic Units or OTUs are detected and the number of OTUs detected is not always easily interpreted in terms of microbial diversity (Fechner et al. 2010b, Bent and Forney 2008)), biofilms with similar profiles are likely to be composed of the same dominant OTUs. A more thorough investigation of the taxonomic composition of biofilm samples, for instance through high-throughput DNA sequencing or a gene-related molecular approach, might provide further information as, for instance, the presence of metal-resistance species or genes within the communities that have high metal tolerance levels. Considering the results obtained here, it would be interesting to study both prokaryotic (using 16S related assays) and eukaryotic (18S) biofilm communities to investigate the impacts of water contamination. Yet DNA sequencing still remains relatively expensive, especially when compared to ARISA which offers a fast and cost-effective means to screen community structure and identify changes in both

384 community structure and diversity in relation with environmental parameters (Magbanua et al.
385 2013). Investigating the presence of metal-resistance genes within the communities would also
386 be an interesting perspective to further explore biofilms' response to metallic contamination.
387

388 **4. Conclusions**

389 This study confirms the importance of both bacterial and eukaryotic microorganisms from
390 river biofilms to investigate the influence of contamination on aquatic ecosystems. Several
391 studies have already pointed at the sensitivity of microbial communities to the influence of
392 contamination, for instance when compared to the eukaryotic macrofauna currently used for
393 water quality monitoring such as fish, algae or macroinvertebrates. River biofilms collected in
394 this study respond to modifications of metallic concentrations in a context of subtle, diffuse
395 contamination, that is to say with low metal concentrations compared to international
396 environmental quality standards. Although tolerance acquisition is not easily interpreted in
397 relation to those particularly low metallic contamination levels, multivariate analysis highlights
398 links between tolerance and metallic exposure. Those results show that a PICT approach is
399 relevant to assess the impacts of low metallic contamination although more data is needed to
400 understand the link between metal speciation *in situ* and metal tolerance of river biofilms.
401 ARISA fingerprints are grouped according to tolerance levels showing that tolerance acquisition
402 is most probably related to modifications of community structure.

403 Both types of biological responses (tolerance acquisition and modifications of community
404 structure) cannot be unequivocally attributed to metal contamination; the individual effects of all
405 environmental parameters that influence biofilm composition and are identified as important in
406 this study cannot be distinguished. Further studies are thus needed to investigate more closely

407 responses to low contamination at the community level, for instance by carefully choosing a
408 sample group of sites with a known range of contamination levels and physico-chemical
409 parameters so that the effects of confounding factors (physico-chemical parameters) can be
410 statistically deduced from water contamination. However, the study confirms that river biofilms
411 have a great potential to be used as sensitive indicators of diffuse, low-level metallic
412 contamination.
413

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422 Supporting information

423 Details about physico-chemical data, metal concentrations and detailed model parameters of
424 the PLS regression are available as Supporting Information.
425

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Table 1. CCU levels of the 13 sampling sites. Cd and Zn tolerance levels (normalized EC_{50} values expressed in mg_{metal}/g_{TSS}) and maximum inhibition levels (%) of biofilms collected at the 13 sites. 95% confidence intervals are shown in brackets.

sampling sites	CCU	Cd tolerance	Cd % max	Zn tolerance	Zn % max
site 1	0.232	8.15 (5.81 - 10.49)	76.43 (72.92 - 80.63)	16.48 (10.90 - 22.06)	74.81 (73.48 - 78.46)
site 2	0.165	3.99 (3.48 - 4.50)	93.30 (92.29 - 94.31)	6.45 (5.32 - 7.57)	89.25 (88.09 - 90.83)
site 3	0.163	3.59 (2.27 - 4.91)	84.98 (81.10 - 90.29)	9.19 (6.08 - 12.29)	77.80 (75.00 - 82.74)
site 4	0.482	3.64 (0.75 - 6.53)	76.17 (76.20 - 81.94)	9.00 (4.72 - 13.27)	73.63 (71.11 - 80.93)
site 5	0.271	2.04 (1.87 - 2.21)	96.00 (95.11 - 96.91)	4.10 (3.43 - 4.78)	93.85 (91.71 - 96.63)
site 6	0.238	2.18 (2.04 - 2.32)	95.47 (94.68 - 96.39)	4.20 (3.84 - 4.55)	88.72 (87.64 - 89.75)
site 7	0.298	1.39 (1.08 - 1.71)	94.60 (92.85 - 96.73)	2.05 (1.60 - 2.51)	87.50 (85.80 - 89.54)
site 8	0.305	2.30 (1.74 - 2.87)	89.78 (89.37 - 9.46)	4.60 (3.69 - 5.50)	86.30 (83.45 - 89.12)
site 9	0.182	4.38 (1.52 - 7.24)	78.65 (76.44 - 80.85)	2.80 (2.15 - 3.45)	81.33 (80.45 - 82.58)
site 10	0.572	4.68 (0 - 12.70)	75.44 (74.12 - 86.24)	10.39 (5.14 - 15.64)	69.30 (67.62 - 74.17)
site 11	0.727	4.21 (2.94 - 5.48)	77.20 (75.93 - 78.60)	4.51 (2.73 - 6.30)	76.74 (73.59 - 81.97)
site 12	0.151	3.69 (2.62 - 4.76)	92.16 (90.17 - 94.34)	3.66 (2.64 - 4.68)	86.23 (85.38 - 87.37)
site 13	0.539	4.99 (3.35 - 6.63)	73.74 (73.06 - 74.64)	3.77 (2.79 - 4.75)	68.17 (66.31 - 70.60)

Table 2. Mean dissolved metal contamination levels at the 13 sampling sites (n=3). Concentrations are expressed in µg/L.

sampling sites	**Cd**	**Cr**	**Co**	**Cu**	**Mn**	**Ni**	**Pb**	**Zn**
site 1	0.01	0.28	0.12	0.75	7.61	0.79	0.11	1.22
site 2	0.01	0.28	0.13	0.64	5.93	0.89	0.06	0.83
site 3	0.01	0.33	0.12	0.50	6.88	0.82	0.15	2.23
site 4	0.01	0.31	0.24	1.40	25.30	1.23	0.19	6.80
site 5	0.01	0.04	0.13	0.89	10.16	0.98	0.06	1.58
site 6	0.01	0.03	0.14	0.89	6.96	1.11	0.10	3.00
site 7	0.01	0.13	0.18	0.84	17.84	1.35	0.06	1.89
site 8	0.01	0.17	0.18	0.95	14.88	1.35	0.12	2.91
site 9	0.01	0.15	0.10	0.37	11.83	0.69	0.07	0.95
site 10	0.04	0.23	0.29	**1.87**	12.60	2.30	0.21	**9.07**
site 11	0.02	0.15	0.40	1.05	49.84	2.37	0.07	5.46
site 12	0.00	0.49	0.15	0.44	7.45	1.19	0.05	2.07
site 13	0.02	1.48	0.53	0.92	15.78	1.49	0.11	**46.65**
mean for all 13 sites	*0.01*	*0.31*	*0.21*	*0.89*	*14.85*	*1.27*	*0.11*	*6.51*
water quality standards								
EU WFD EQSs[1]	0.25			1.4		4	1.2	7.8
US EPA CCCs[2]	0.19			26		150	19.65	298.64

[1] European Union Water Framework Directive (EU WFD) EQSs are given for waters with CaCO3 > 200 mg/L. Water hardness varies between 173 and 351 mg/L CaCO3 (only site 1 has a water hardness below 200 mg/)L.

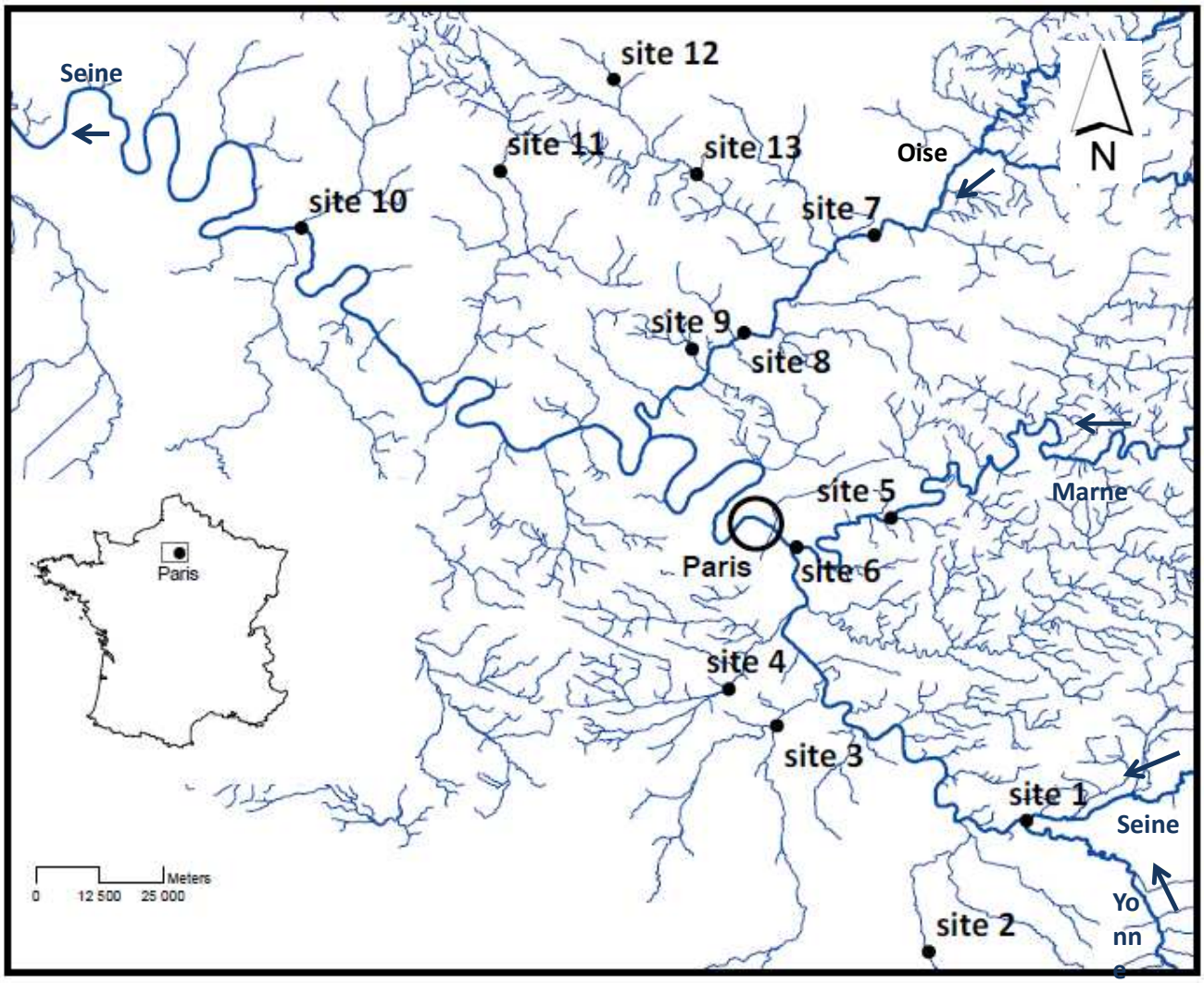
[2] CCCs (Criterion Continuous Concentrations) were corrected using hardness - dependent parameters from Appendix B of the National Recommended Water Quality Criteria from the EPA. The CCCs given here are those obtained for site 9 which has the highest water hardness (351 mg/L $CaCO_3$).

Values indicated in bold correspond to dissolved metal concentrations that are above the corresponding EQSs. Values indicated in italics are mean concentrations for the 13 sites.

Table 3. Variable importance (Variable Influence in the Projection) in the PLS model and corresponding regression coefficients for each Y variable: Cd tolerance, Zn tolerance and maximum % inhibition of β-glucosidase activity obtained with Cd and Zn. Only variables with VIP > 0.8 are represented. Metal concentrations are indicated by 'lab' for labile. 'dis' for dissolved and 'tot' for total metal concentrations.

Variable	VIP	CoefftolCd	CoefftolZn	Coeffmax%Cd	Coeffmax%Zn
pH	1.442	-0.062	-0.139	0.036	0.085
POC	1.435	0.152	0.149	-0.165	-0.133
dis Cr	1.329	0.126	0.054	-0.107	-0.140
NH_4^+	1.327	-0.231	-0.112	0.236	0.144
dis Pb	1.314	0.066	0.164	-0.116	-0.120
tot Pb	1.217	0.080	0.112	-0.125	-0.132
tot Zn	1.138	0.101	0.024	-0.106	-0.136
Chl-a	1.129	-0.169	-0.053	0.163	0.099
lab Ni	1.074	-0.132	-0.043	0.104	0.102
lab Cr	1.061	-0.021	-0.122	0.010	0.005
Na^+	1.050	-0.084	-0.069	0.052	0.037
Alkalinity	1.032	-0.052	-0.120	0.042	0.037
DW	1.032	-0.075	-0.083	0.047	0.021
Mg^{2+}	1.018	-0.034	-0.039	-0.042	0.029
dis Zn	1.014	0.028	0.016	-0.027	-0.082
tot Cr	1.014	0.133	-0.016	-0.147	-0.132
tot Cd	1.006	0.082	-0.005	-0.074	-0.092
tot Co	0.985	0.061	-0.035	-0.067	-0.061
dis Co	0.980	0.027	-0.005	-0.019	-0.055
tot Ni	0.976	-0.030	-0.064	0.026	0.022
AFDW	0.953	-0.118	-0.052	0.083	0.050
dis Ni	0.950	-0.084	-0.024	0.097	0.047
lab Co	0.930	0.004	-0.042	-0.048	-0.020
tot Cu	0.929	0.101	0.089	-0.158	-0.107
SO_4^{2-}	0.924	0.026	-0.036	-0.060	-0.038
tot Mn	0.920	0.073	0.017	-0.128	-0.065
dis Cu	0.914	-0.069	0.058	0.059	0.019
Cl^-	0.906	-0.044	-0.035	0.024	0.009

Figure 1: Map of the sampling sites in the Seine river basin, North of France.



The location of the city of Paris is indicated by a circle. The names of main rivers are pointed out and arrows show the direction of the flow.

Fig. 2.

PCA score plot (a) and loading plot (b) of the environmental parameters (physico-chemical data and total, dissolved and labile metal contamination data) of the 13 sites. Metal concentrations are indicated by ‘lab’ for labile, ‘Diss’ for dissolved and ‘tot’ for total metal concentrations.

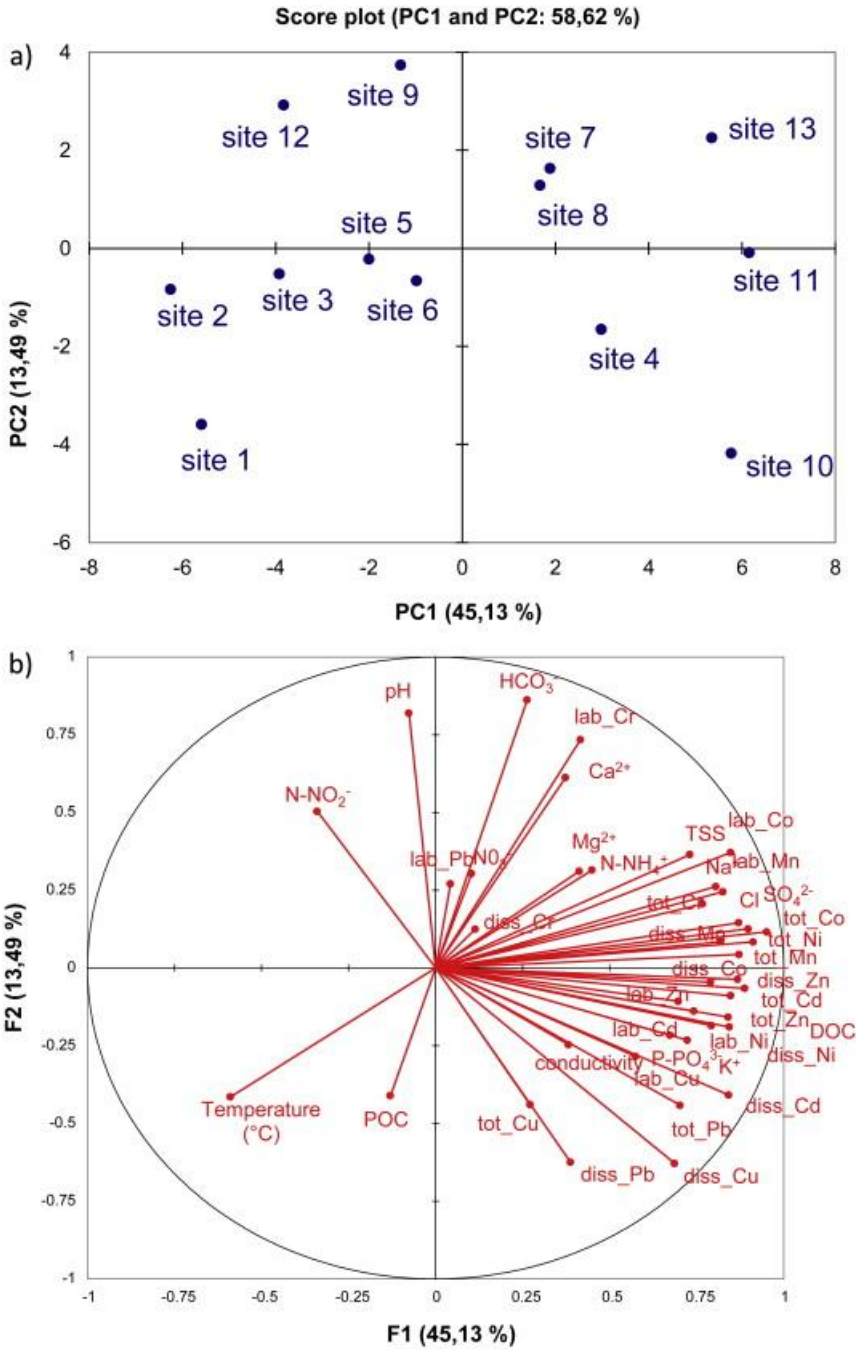
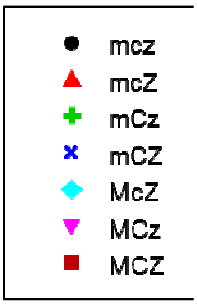


Figure 3. 2D projections (along the first two components) of 3D-nMDS plots of bacterial (a) and eukaryotic (b) ARISA fingerprints (stress values are 3.58 % (a) and 4.86%. (b) for 3D plots).

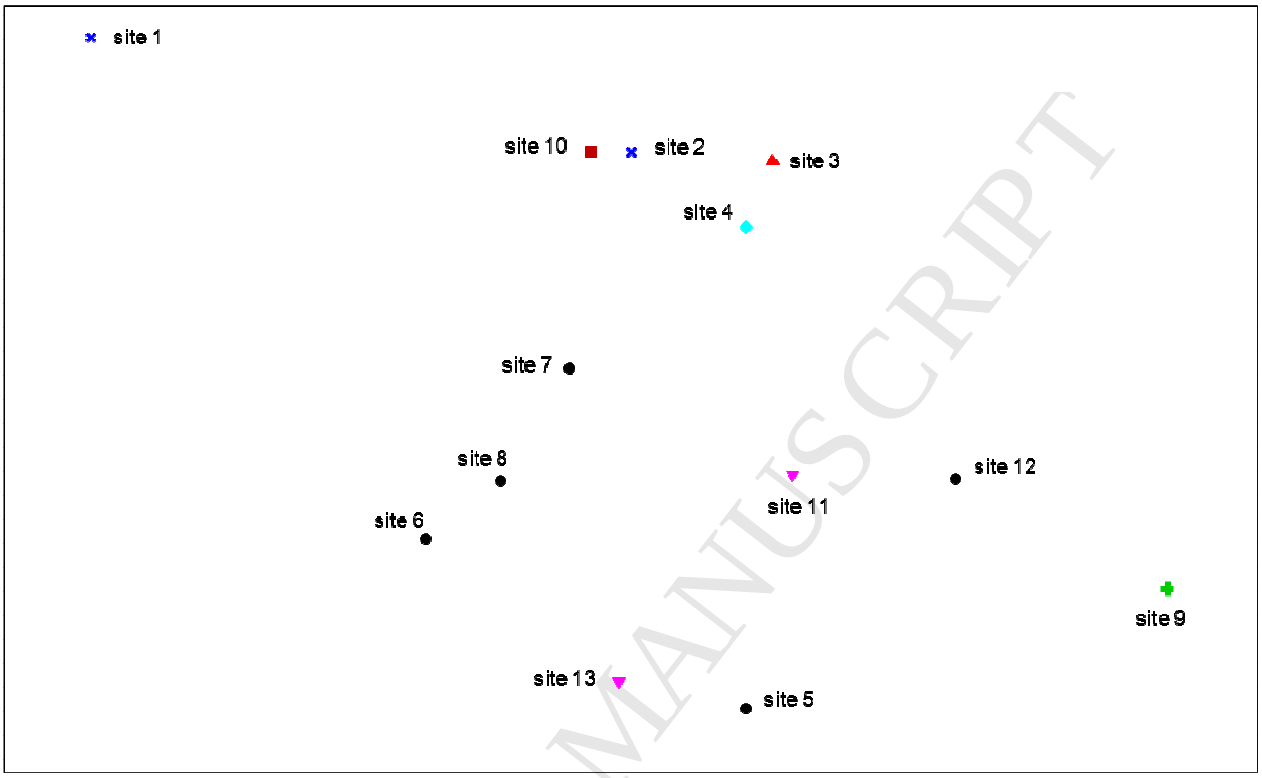
Each ARISA profile (biofilm sample) is characterized by three parameters (CCU of the sampling site, Cd and Zn biofilm tolerance), each represented as a lower-case letter (m, c and z) or capital letter (M, C and Z) whether the corresponding parameter is above or below the mean calculated for all 13 sites.

Legend:

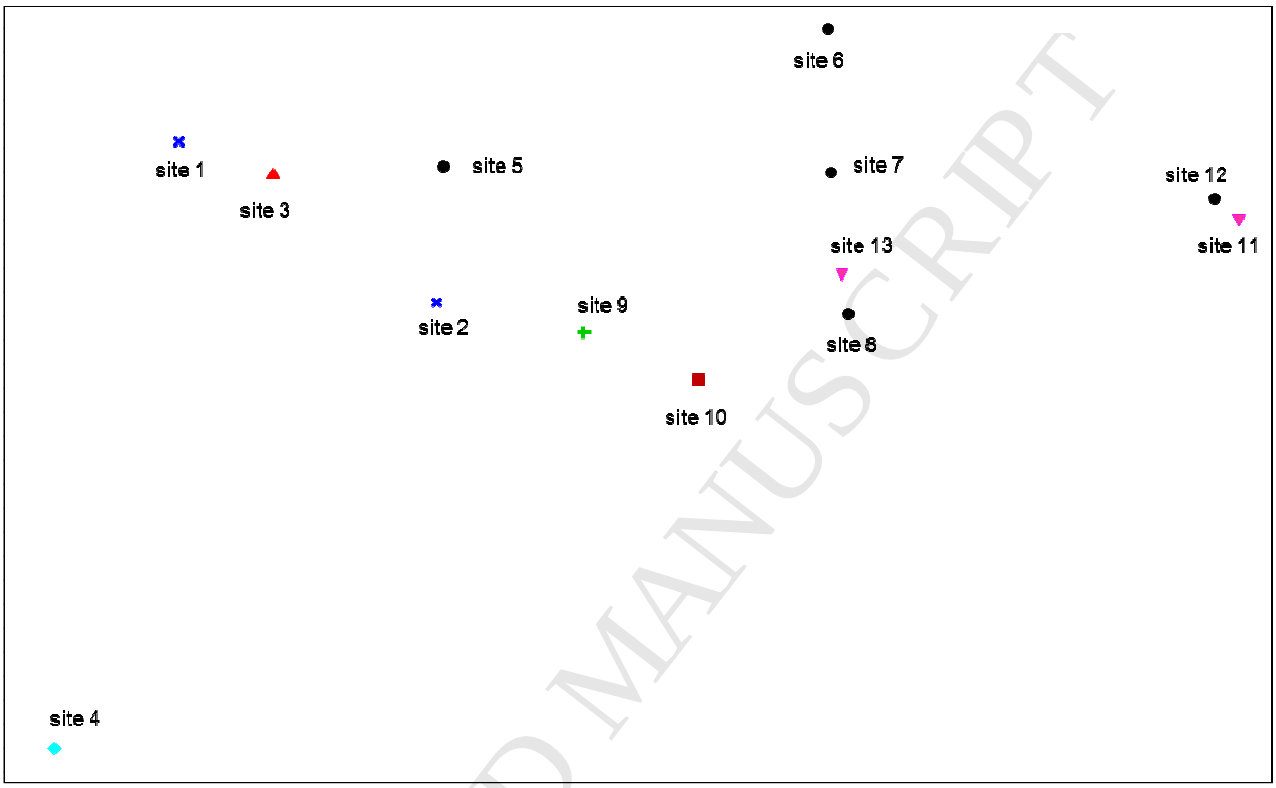


	Low	high
CCU	m	M
Cd tolerance	c	C
Zn tolerance	z	Z

a



b



ARISA profiles of biofilms with high Zn tolerance levels (Z) are represented by: ▲, ×, ■, ♦.

ARISA profiles of biofilms with high Cd tolerance levels (C) are represented by: ■ ▼× +.

ARISA profiles of biofilms collected at sites with high CCU (M) are represented by: ■ ▼ ♦.

Highlights:

River biofilms were collected on 13 sites with low metallic contamination levels

Multivariate analysis shows that biofilm metal tolerance was linked to contamination

Environmental parameters also modulate biofilm tolerance levels

Biofilms with similar tolerance levels have similar ARISA profiles

Biofilms are potential sensitive indicators of diffuse, low-level contamination

Supporting information

Linking community tolerance and structure with low metallic contamination: a field study on 13 biofilms sampled across the Seine river basin.

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Table S1. Mean values (n=3) of physico-chemical parameters of river water at the 13 sampling sites. Ionic concentrations and Total Suspended Solids in river water (TSSw) are expressed in mg/L, conductivity in µS/cm, DOC in mgC/L and POC in mgC/gTSS. nd: not determined.

sampling sites	HCO_3^-	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	SO_4^{2-}	$N0_3^-$	$P\text{-}PO_4^{3-}$	$N\text{-}NO_2^-$	$N\text{-}NH_4^+$	T (℃)	pH	conductivity	TSS	DOC	POC
site 1	168.8	7.0	2.1	2.9	64.3	13.1	11.1	11.6	0.15	0.02	nd	21.7	7.71	699.3	4.6	2.3	131.0
site 2	233.8	12.4	3.2	2.8	92.7	28.0	16.2	22.5	0.14	0.04	nd	18.8	7.74	553.3	4.2	2.5	139.9
site 3	239.9	11.1	3.2	5.6	93.2	24.6	18.0	27.5	0.15	0.08	nd	16.3	7.70	943.7	12.0	2.0	137.5
site 4	213.5	19.4	5.1	8.6	84.3	37.2	38.4	21.0	0.29	0.04	0.04	16.1	7.71	574.7	16.9	3.8	108.3
site 5	197.2	14.6	2.4	9.2	69.8	19.9	28.7	7.9	0.16	0.05	0.06	17.5	7.89	1178.3	9.5	2.8	40.6
site 6	201.3	16.1	2.8	9.6	71.8	22.5	34.3	8.8	0.17	0.06	0.04	17.7	7.85	1386.7	13.2	2.8	56.5
site 7	280.6	31.1	5.4	10.2	102.5	41.4	48.8	18.2	0.17	0.04	0.09	16.8	7.93	1158.7	13.4	3.0	25.2
site 8	278.6	32.4	5.0	10.2	100.7	41.2	52.3	18.7	0.18	0.04	0.06	15.7	7.99	631.3	8.3	3.1	78.8
site 9	307.0	17.4	2.2	20.4	107.0	34.1	51.0	29.8	0.16	0.06	nd	12.6	8.00	670.3	20.6	2.5	48.6
site 10	211.5	21.3	5.5	8.3	85.5	56.7	68.3	25.7	0.24	nd	0.02	11.7	7.58	1494.3	12.3	5.9	65.1
site 11	250.1	25.8	7.9	6.5	97.7	60.7	81.5	16.0	0.22	0.01	0.01	9.1	7.76	1952.7	34.5	10.6	97.9
site 12	278.6	7.8	2.0	3.6	86.5	22.0	9.2	27.6	0.19	0.02	0.06	9.7	7.86	802.7	10.6	2.7	80.8
site 13	284.7	31.2	3.8	4.7	97.1	47.0	81.0	21.7	0.18	0.08	0.11	10.3	7.83	599.7	18.7	4.2	123.0

Table S2. Parameters used Parameters for Calculating Freshwater Dissolved Metals Criteria That Are Hardness-Dependent according to US EPA.

metal	mC	bC	conversion factor
Cd	0.7409	-4.719	1.101672 × (Ln([hardness]) × 0.04838)
Cu	0.8545	-1.702	0.96
Pb	1.273	-4.705	1.46203 × (Ln([hardness]) × 0.145712)
Ni	0.846	0.0584	0.997
Zn	0.8473	0.884	0.86

Source: National Recommended Water Quality Criteria, http://water.epa.gov/scitech/swguidance/standards/criteria/current/index.cf

Table S3. Physico-chemical parameters of Montdore mineral waters (in mg/L)

HCO_3^-	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	SO_4^{2-}	$N0_3^-$
25.8	2.7	0.9	1.7	4.1	0.9	1.14	0.8

Table S4. Mean total (a) and labile (b) metal contamination levels at the 13 sampling sites (n=3).

Concentrations are expressed in µg/L.

a.

Sampling sites	Cd	Cr	Co	Cu	Mn	Ni	Pb	Zn
site 1	0.03	0.55	0.15	7.05	16.41	1.17	1.09	6.71
site 2	0.02	0.07	0.14	1.61	11.24	1.24	0.48	2.69
site 3	0.03	0.30	0.13	3.53	17.92	1.06	1.74	7.21
site 4	0.05	1.30	0.43	6.08	42.49	2.01	2.58	16.01
site 5	0.03	0.49	0.21	3.09	17.11	1.65	0.66	4.40
site 6	0.04	0.89	0.27	4.99	20.46	1.88	1.39	8.36
site 7	0.04	0.96	0.39	6.43	35.29	2.63	1.10	11.17
site 8	0.04	0.64	0.31	4.76	27.80	2.32	1.15	7.63
site 9	0.04	1.14	0.32	3.46	27.66	1.90	0.89	5.84
site 10	0.09	1.75	0.67	4.59	31.62	3.41	3.42	26.14
site 11	0.06	1.23	1.01	2.66	111.02	3.49	1.41	15.32
site 12	0.04	0.87	0.25	0.88	12.91	1.75	0.51	4.89
site 13	0.07	6.12	0.88	2.85	39.42	2.36	2.01	60.03

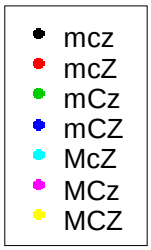
b.

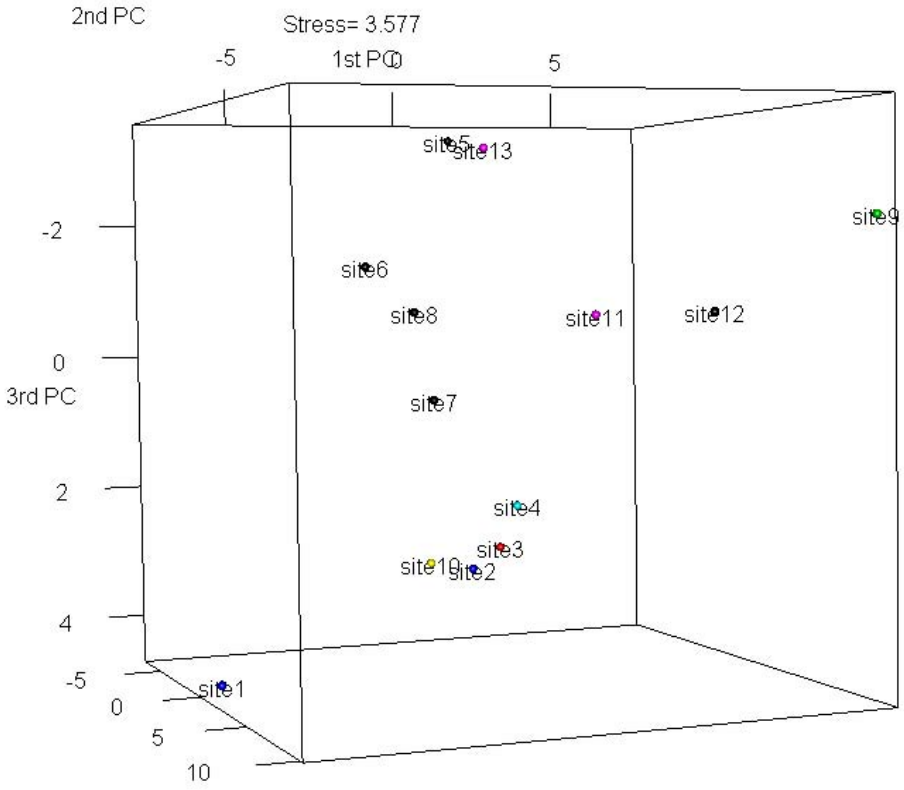
Sampling sites	Cd	Cr	Co	Cu	Mn	Ni	Pb	Zn
site 1	0.001	0.02	0.01	0.11	3.22	0.12	0.02	0.92
site 2	0.001	0.01	0.00	0.09	1.81	0.16	0.03	0.49
site 3	0.002	0.03	0.01	0.10	2.67	0.10	0.13	1.08
site 4	0.002	0.04	0.04	0.30	14.02	0.29	0.13	1.90
site 5	0.002	0.08	0.03	0.24	8.23	0.58	0.05	0.85
site 6	0.002	0.10	0.02	0.19	4.27	0.22	0.08	2.05
site 7	0.003	0.09	0.03	0.19	11.78	0.45	0.04	1.66
site 8	0.005	0.18	0.04	0.25	7.55	0.36	0.09	2.10
site 9	0.002	0.12	0.03	0.18	6.09	0.16	0.03	1.08
site 10	0.009	0.02	0.03	0.38	5.36	0.50	0.02	2.94
site 11	0.002	0.05	0.10	0.10	25.94	0.57	0.03	1.03
site 12	0.001	0.07	0.03	0.05	5.34	0.09	0.04	0.22
site 13	0.002	0.51	0.13	0.17	12.13	0.27	0.11	8.78

Figure S1. 3D MDS of ARISA bacterial (a) and eukaryotic (b) profiles. Stress values are 3.58% (a) and 4.86%. (b). Each fingerprint (biofilm sample) is characterized by three parameters (CCU of the sampling site, Cd and Zn biofilm tolerance), each represented as a lower-case letter (m, c and z) or capital letter (M, C and Z) whether the corresponding parameter is above or below the mean calculated for all 13 sites. For each biofilm sample (sampling site) ARISA profils were obtained in triplicates.

Legend of the 3-dimensional plot:

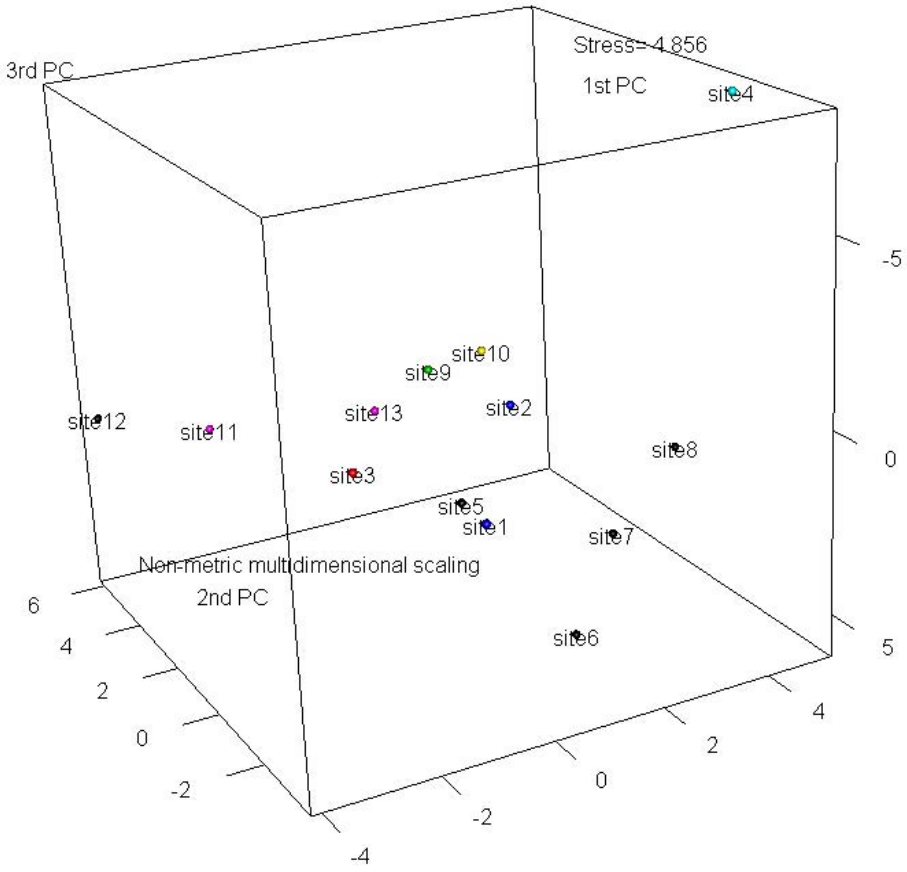
	Low	high
CCU	m	M
Cd tolerance	c	C
Zn tolerance	z	Z





Non-metric multidimensional scaling

a



b