

Low exposure levels of urban metals induce heterotrophic community tolerance: a microcosm validation

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Abstract The biological response of periphyton chronically exposed to metals of urban origin (Cd, Ni and Zn) was investigated with a Pollution-Induced Community Tolerance (PICT) approach using a previously developed short-term toxicity test based on β -glucosidase (heterotrophic) activity. Periphyton was grown on plastic membranes immersed in indoor aquaria contaminated with metals at realistic contamination levels (0.3, 3 $\mu\text{g/l}$ for Cd, 5, 50 $\mu\text{g/l}$ for Ni, 20, 200 $\mu\text{g/l}$ for Zn). After 3 weeks of exposure, biofilms' parameters (dry-weight, chlorophyll *a* concentration, heterotrophic activity) were analyzed and tolerance acquisition of the heterotrophic communities was assessed using the toxicity test. Modifications of bacterial and eukaryotic community structure were assessed with Automated Ribosomal Intergenic Spacer Analysis (ARISA). Effects of metal exposure were observed on biofilms parameters in the Cd and Zn experiments. Tolerance levels increased for both Cd-exposed biofilms, and for the high metal treatment biofilms in the Ni and Zn experiments. Analysis of the ARISA profiles showed that metal exposure affected the structure of both bacterial and eukaryotic

communities. Moreover, Cd tolerance of the Zn-exposed heterotrophic communities was evaluated, which showed that the Zn-tolerant community (high metal treatment in the Zn experiment) also became tolerant to Cd (co-tolerance). The study shows that tolerance acquisition can be detected after exposure to environmental metal concentrations using β -glucosidase activity as an endpoint in short-term toxicity tests.

Keywords Biofilms · Urban metal · Community tolerance · ARISA

Introduction

Urban pollution is characterized by a mixture of contaminants at low and sub-lethal levels (Thévenot et al. 2007; Tusseau-Vuillemin et al. 2007). Linking urban chemical contamination to biological impairment is therefore a complex task as the effects of subtle, chronic contaminations do not engender easily-detectable biological responses (Bourgeault et al. 2010). The Pollution-Induced Community Tolerance (PICT) concept was proposed by Blanck et al. (1988) as a new ecotoxicological approach using tolerance acquisition at the community level to detect a biological response to a toxic exposure. Increased community tolerance to a toxic agent is indeed assumed to be the consequence of a selection pressure on the community caused by the toxic agent (Clements and Rohr 2009). PICT is induced either by selection of more tolerant species or by phenotypic or genotypic adaptation of already present species (Blanck 2002).

Periphyton (or biofilm) is composed of both autotrophic and heterotrophic microorganisms embedded in a matrix of polysaccharides. It develops on immersed substrata like

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rocks, macrophytes or sediment. In streams and rivers, periphyton is often the first biological entity to respond and recover from stress (Burns and Ryder 2001; Sabater et al. 2007). It also plays an important part in nutrient and energetic cycles in aquatic systems. Collected in situ on artificial substrata, biofilms provide a representative sample of the natural community and an indication of the overall health of aquatic ecosystems (Sabater et al. 2007). They have indeed been successfully used to identify PICT effects with herbicides, antifouling agents or metals (Blanck 2002; Sabater et al. 2007).

The crucial feature of the PICT concept is that community tolerance is determined by the means of short-term toxicity tests: comparison of the obtained EC_{50} value with that of an unexposed (reference) community clarifies whether or not the toxicant has affected the community (Blanck and Dahl 1996). Most studies have focused on the impact of toxicants (metals, pesticides, etc.) on the algal component of periphyton or phytoplankton using short-term photosynthetic toxicity tests to bring to light tolerance induction (for instance Dorigo et al. (2010) or Serra et al. (2010)). Moreover, the toxicity tests are usually performed with the use of radioactive reagents, like incorporation of labelled-thymidine for bacterial growth and labelled- CO_2 for photosynthesis (see for instance Blanck 2002, Blanck et al. 2003). In this study, a recently-developed short-term toxicity test, based on β -glucosidase activity (Fechner et al. 2010a) was used for comparative evaluation of tolerance levels of heterotrophic communities exposed to urban metals. Indeed exo-enzymes control essential metabolic pathways in aquatic ecosystems and are thus interesting to study the effects of environmental disruptions on the functioning of aquatic ecosystems (Admiraal and Tubbing 1991). In particular, β -glucosidase represents the global activity of heterotrophic microorganisms (Chrost 1991). To our knowledge, the impact of metals of urban origin on heterotrophic communities has not yet been studied using a PICT approach.

PICT is a sign that the community under study has been affected and is therefore usually accompanied by changes in biofilm community structure. These modifications can be assessed by the use of indicators like biofilm biomass, species richness or the abundance of particular taxa (Blanck 2002). More recently, modifications of community structure have been identified using fingerprinting techniques like DGGE (Dorigo et al. 2010), or ARISA (Fechner et al. 2010b).

The aim of this study is to test the sensitivity of tolerance measurements obtained with the β -glucosidase toxicity test to assess the impact of metallic exposure at realistic, environmental concentrations. Periphyton was thus exposed to metals that are typical of urban contamination (Cd, Ni and Zn) under controlled conditions in

microcosms. ARISA fingerprints of the communities were also used to assess modifications of bacterial and eukaryotic community structures under metallic exposure.

Materials and methods

Periphyton was exposed to metals in three separate microcosm experiments (long-term metal exposure to either Cd, Ni or Zn). For each experiment, a natural river biofilm (biofilm inoculum) was used as inoculum for the microcosms. At the end of the long-term exposure, the metal tolerance levels of biofilms from the microcosms were assessed using a short-term toxicity test based on β -glucosidase activity and modifications of both bacterial and eukaryotic community structures were assessed using the ARISA technique.

Biofilm inoculum

Periphyton was grown on Low Density PolyEthylene (LDPE) plastic membranes immersed in a third-order river: the Rémarde river (mean annual flow in 2008: $0.5 \text{ m}^3/\text{s}$) which is relatively unaffected by the urban area of Paris in terms of metallic contamination (Table S1 in Supplementary material). After a colonization time of at least 50 days (50, 56 and 82 days for the Cd, Ni and Zn experiments respectively), colonized membranes were brought back to the laboratory in 250 ml glass bottles filled with mineral water (Valvert, France). Membranes were carefully hand-scraped to remove periphyton and make a periphyton suspension in Valvert mineral water. This periphyton suspension was used to inoculate the microcosms at the beginning of each experiment, therefore a new inoculum (river biofilm) was collected for each metal exposure experiment: in October 08 for the Cd experiment, November 08 for the Ni experiment and in February 09 for the Zn experiment. Aliquots of the three river biofilms were also centrifuged and stored at -80°C for ARISA fingerprinting (see below).

Long-term metal exposure of biofilms in microcosms

The medium used to cultivate periphyton in the microcosms was Valvert mineral water (Valvert, France) enriched with nutrients. Nutrients (N, P, Si) were added as $\text{Ca}(\text{H}_2\text{PO}_4)_2$, $\text{Ca}(\text{NO}_3)_2$ and Na_2SiO_3 at concentrations of 1 mg/l PO_4^{3-} , 20 mg/l NO_3^- and 32 mg/l SiO_2 , which were close to natural nutrient concentrations measured in the Rémarde river at the inoculum sampling site. Valvert mineral water was chosen for the medium so that physico-chemical parameters were similar to those from the Rémarde river (Table S2 in Supplementary material).

In each experiment, periphyton grew on clean immersed LDPE membranes (140 cm², 14 membranes per aquarium) at 20°C, under continuous water mixing conditions using water pumps (Eheim Compact 300, 300 l/h) and a light regime of 13 h/11 h light/dark cycle using neon lamps (Sylvania, Gro-Lux, F14W-GRO-T8) during 3 weeks. The medium was changed weekly during the 3 weeks of colonization.

At the beginning of each experiment, a stock solution of metal was prepared by adding CdNO₃, NiSO₄ or ZnSO₄ to distilled water for respectively the Cd, Ni and Zn experiments. Prior to the beginning of the experiments, indoor plastic aquaria and LDPE membranes were rinsed with 10% HNO₃, then twice with distilled water, and finally left to equilibrate for 24 h with the cultivation medium spiked with metals to limit adsorption of metals on the containers' walls.

Periphyton was exposed to each metal in separate experiments (Cd, Ni and Zn) for 3 weeks. In each experiment, two metal concentrations ("low metal" and "high metal") were tested with one control (aquaria filled with metal-free medium), with one replicate microcosm per treatment. Periphyton was exposed to Cd at 0.3 and 3 µg/l, Ni at 5 and 50 µg/l and Zn at 20 and 200 µg/l in the Cd, Ni and Zn experiments respectively. Metal concentrations in the aquaria were measured daily in aliquots of 5 ml of aquaria water: the decrease in metal concentration, mainly due to metal absorption by periphyton, was corrected by adding stock solutions of metal as required (≤ 100 µl per aquarium).

Laboratory analyses

Water samples were collected daily from the aquaria for measurement of physico-chemical parameters and metal concentrations. Metal concentrations were measured daily on 5-ml samples acidified to 1% HNO₃ (suprapure, Merck) by graphite furnace Atomic Absorption Spectrometry (AAS) for the Cd and Ni experiments (SpectrAA 220Z, GTA 110Z, Varian Inc., USA), flame AAS for the Zn experiment (SpectrAA 220FS, Varian Inc., USA). Validity of the method was checked daily with a certified reference material (EP-L-2, SCP Science). Mean standard deviations from the certified values were 2.5% (Cd), 2.3% (Ni), and 10% (Zn).

General biofilm parameters

After 3-weeks of biofilm growth, the colonized membranes were scraped in a known volume of mineral water (Montdore, Grand Barbier, France) as in (Fechner et al. 2010a) to form a single biofilm sample per aquarium which was mixed by magnetic stirring. Aliquots of biofilm

suspension were assigned to various analyses in terms of biomass, metal accumulation and tolerance acquisition. Periphyton dry weight (DW), ash-free dry weight (AFDW) and chlorophyll *a* concentration were determined as in Fechner et al. (2010b). To perform ARISA, aliquots of 50 ml of each periphyton suspension (biofilms from the microcosms at the end of the three experiments and river biofilm at the beginning of each experiment) were centrifuged for 15 min at 10,000×*g*, and 4°C. Supernatants were discarded and pellets were frozen and stored at -80°C for further use. Metal accumulated in the biofilms was measured according to Meylan et al. (2004). Aliquots of biofilm suspensions (approximately 20 ml) were filtered on 0.45 µm PES filters (47 mm diameter, Sartorius Biolab Products, Sartorius Stedim Biotech, Germany) previously washed by immersion in 10% HNO₃ (suprapure, Merck, France) for 48 h and then rinsed twice with ultrapure water. For each biofilm, an aliquot of 20 ml was also washed with 4 mM EDTA (final concentration) for 10 min to remove metal adsorbed to cell walls and most of the inorganic complexes and thus obtain a measure of the intracellular metal content of periphyton. Other samples (not washed with EDTA) were used to obtain a measurement of total metal content of periphyton. Filters were then digested on a *DigiPREP* Jr. (SCP Science, France): 4 ml HNO₃ (65% suprapur, Merck, France) were added to the filters in 50 ml tubes (SCP Science, France) which were heated to 100°C for 2 h, then 2 ml H₂O₂ (suprapur, Merck, France) were added to the tubes which were again heated to 100°C for 2 h. After dilution to 50 ml with milli-Q water, the metal concentrations of the samples were measured by AAS (Varian Inc., USA).

β-Glucosidase short-term toxicity test

Biofilm metal tolerance was assessed by the means of a heterotrophic acute toxicity test using β-glucosidase activity (Fechner et al. 2010a). Briefly, biofilms were exposed for 1 h to acute levels of metals (at least six concentrations of metal varying between 0.001 and 10 mM were tested in triplicates 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 or MUF-GLU (Sigma-Aldrich). Fluorescence of 4-methylumbelliferone (or MUF) was measured using either a SAFAS SP 2000 Xenius spectrofluorimeter (Safas SA, Monaco, France) (excitation: 348 nm and emission: 448 nm) for the Cd experiment, or a LB 941 Tristar Ti fluorescence microplate reader (Berthold Technologies, Bad Wildbad, Germany) (excitation/emission filters: 355 and 460 nm) in

the Ni and Zn experiments. Fluorescence measurements were converted into MUF concentrations by calibrating the spectrofluorimeter with a range of MUF solutions prepared in demineralised water. % inhibition of β -glucosidase activity was then calculated using MUF concentrations measured for each metal concentration tested. 50% effect concentrations (EC_{50}) and maximum inhibition levels were determined from the dose/effect curves, which 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 (Hill 1910; Vindimian 2001). The general form of the curve fitted on the data is:

$$F(x) = 100 \times \frac{(p_0 - p_{\max})}{p_0} \times \frac{x^H}{x^H + EC_{50}^H}$$

where x is the metal concentration, p_0 is the effect observed without toxicant, p_{\max} is the predicted effect when the toxicant concentration is infinite, and H is the Hill number (slope of the toxicity curve). Each parameter (p_0 , p_{\max} , EC_{50} and H) was adjusted for the calculation by non-linear regression. 95% confidence intervals around the fitted parameters were estimated using a Bootstrap method. Parameters (EC_{50} values and maximum % inhibition) were considered significantly different when 95% confidence intervals were disjointed.

Community tolerance to metal was assessed by calculation of a normalized EC_{50} value by dividing the EC_{50} value by the Total Suspended Solids (TSS) concentration of the periphyton suspension used for the short-term test (Fechner et al. 2010a) and the maximum inhibition level was calculated with the adjusted parameters p_0 and p_{\max} .

In each experiment, the short-term toxicity test was used to assess the tolerance levels of the microcosms' biofilms (control, low and high metal treatments) to the metal used for the long-term exposure. Moreover, in the Zn experiment, the tolerance to Cd of the microcosms' biofilms (long-term exposure to Zn: control, low and high metal treatments) was also assessed (short-term toxicity test with Cd).

ARISA fingerprinting of periphyton

DNA was extracted from the thawed biofilm pellets (river biofilms and biofilms from the microcosms) using the Power Soil DNA Isolation Kit (Mobio Laboratories, Inc., Carlsbad, US) according to the manufacturer's instructions. DNA amplification and ARISA fingerprinting were performed as in Fechner et al. (2010b). Briefly, primers ITSf/ITSr (Cardinale et al. 2004) were used for amplification of bacterial 16S–23S intergenic spacers and primers 2234C/3126T (Ranjard et al. 2001) were used for amplification of eukaryotic ITS1–5.8S–ITS2 regions. PCR-amplified fragments were separated on an electrophoresis Bioanalyzer (2100 Electrophoresis Bioanalyzer, Agilent

Technologies, Santa Clara, CA) and fluorescence data was converted into electrophoregrams using 2100 Expert software (Agilent Technologies, Santa Clara, CA, US).

Comparisons between ARISA fingerprints (either bacterial or eukaryotic) were made using the Jaccard index as a % of similarity (Legendre and Legendre 1998) between two fingerprints from the same experiment using the 2100 Expert software (Agilent Technologies, Santa Clara, CA, US):

$$D(i, j) = a / (a + b + c),$$

where a is the number of fragments contained in both samples, b number of fragments contained in sample i but not j , and c is the number of fragments contained in sample j but not i . This allows comparison between fingerprints using solely the presence or absence of fluorescence peaks and without taking into account peak intensity. To include the maximum number of peaks while excluding background fluorescence, only peaks with a fluorescence value superior to 20 FU (Fluorescence Units) were considered for calculation of the Jaccard indexes. ARISA fragment lengths differing by more than 3 bp were considered distinct.

Electrophoregrams in ASCII formats were also imported into the StatFingerprints R package (Michelland et al. 2009) using the 2.10.1 version of the R project. ARISA profiles were aligned and the area under each curve was normalized to 1 before calculation of the Shannon and evenness indexes, to assess community diversity using the area under peaks. For calculation of the Shannon and evenness indexes, all fluorescence peaks were considered (no fluorescence threshold was fixed as in the calculation of the Jaccard indexes).

Results

Biofilm long-term metal exposure in the microcosms

Metal concentrations in the microcosms' media were measured daily before and after spiking to readjust metal exposure levels. Mean metal concentrations remained close to the expected values (Table S3 in Supplementary material). Exposure levels had to be compensated for every day, especially for low metal treatments. Levels of total and intracellular metal accumulated in the biofilms (Table 1) confirm that biofilms accumulated metal during exposure. Cd and Ni concentrations in the control microcosms were always below the detection level for the Cd and Ni experiments respectively. As regards the Zn experiment, Zn could be detected in the control microcosm, with a mean of 1.3 $\mu\text{g/l}$ corresponding to the Zn concentration initially detected in Valvert water.

Table 1 Impact of long-term metal exposure on biofilm parameters (dry weight, chlorophyll *a* concentration (Chl-*a*) and β -glucosidase activity) and concentrations of total and intracellular metals in the biofilms

Long-term exposure	Metal treatment	Biofilm parameters			Biofilm-accumulated metals		
		DW	Chl <i>a</i>	β -Glucosidase activity	Total	Intracellular	Total/ intracellular
Cd	Low metal	88.4	113	86.3	13.0	19.4	1.4
	High metal	60.9 ^a	46	32.1 ^a	127.0	183.4	1.5
Ni	Low metal	96.0	176	63.0 ^a	10.2	6.4	1.5
	High metal	73.1	111	89.5	77.2	51.7	1.4
Zn	Low metal	106.7	118	35.7	3.5	6.1	2.2
	High metal	10.2 ^a	13	0.7 ^a	35.7	37.2	3.6

Metal-induced variations of each biofilm parameter are expressed as percentages of the value corresponding to the control biofilm for that parameter. Total and intracellular metal concentrations accumulated in the metal-exposed biofilms (low and high metal treatments) are expressed as ratios calculated from the metal concentration (either total or intracellular) measured in the corresponding control biofilms for each experiment. Ratios between total and intracellular metal concentrations for each biofilm samples are also shown. ^a A significant decrease from the value corresponding to the control biofilm. Statistical differences between the three biofilms (control, low and high metal treatments) from each experiment were assessed using a non-parametric test (Kruskal–Wallis followed by the Dunn test for pairwise comparisons of means) as few replicates were measured (n = 2 for DW values and n = 3 for β -glucosidase activity; no statistical analysis of the Chl-*a* and metal concentrations was performed as n = 1 for those parameters)

Periphyton characterization

Variations of biofilm parameters (dry weight or DW, chlorophyll *a* or Chl-*a* concentration and mean β -glucosidase activity) were observed with increasing metal exposure (Table 1). Mean DW decrease reached 39, 27 and 90% respectively in the Cd, Ni and Zn experiments for the high metal exposed biofilms, and was not significant for the low metal treatment biofilms. Chl-*a* concentrations decreased only for the high metal treatments in both the Cd and Zn experiments. Heterotrophic activity (β -glucosidase) decreased with metal exposure in the Cd and Zn experiments. In the Ni experiment, heterotrophic activity decreased for the low metal treatment.

Variations of ARISA profiles (Fig. S1 in Supplementary material) with metal exposure were observed for both bacterial and eukaryotic communities. Bacterial ARISA profiles showed a decrease of the number of fluorescence

peaks with increasing metal exposure for all experiments (Table 2). Both Shannon index and evenness (Table 2) computed with bacterial ARISA profiles of the control biofilms were always higher than for metal-exposed communities. They also varied between metal exposure levels.

Eukaryotic ARISA profiles also showed modifications of eukaryotic community structures with metal exposure. In all experiments, both the Shannon indexes and evenness are higher for the high metal treatments than for the other biofilms.

ARISA fingerprints of the microcosms' biofilms can also be compared to the fingerprints obtained with the river biofilm (inoculum from the Rémarde river collected in situ at the beginning of each experiment). With both bacterial and eukaryotic ARISA profiles, Shannon indexes are higher for the Rémarde biofilm used as inoculum in each experiment, except for the eukaryotic profiles in the Ni experiment.

Table 2 Shannon-Weaver indexes (S), evenness (E) and number of major fluorescence peaks (fluorescence > 20 FU) from the ARISA profiles of inoculum (river biofilm), control, low and high metal treatment biofilms from the Cd, Ni and Zn experiments

	Cd experiment						Ni experiment						Zn experiment					
	Bacteria			Eukaryotes			Bacteria			Eukaryotes			Bacteria			Eukaryotes		
	S	E	Number of peaks	S	E	Number of peaks	S	E	Number of peaks	S	E	Number of peaks	S	E	Number of peaks	S	E	Number of peaks
Inoculum	3.3	1.1	4	2.5	1.1	6	3.8	1.3	5	1.0	0.6	4	3.2	1.0	2	2.0	0.8	6
Control	2.0	0.8	6	1.7	0.7	7	2.1	0.8	5	1.6	0.6	6	1.8	0.7	6	1.6	0.6	4
Low metal	1.0	0.4	3	1.7	0.7	7	1.5	0.6	5	1.2	0.6	5	1.0	0.5	5	1.5	0.6	6
High metal	1.5	0.6	4	1.8	0.8	8	0.8	0.4	2	2.0	0.8	8	1.8	0.7	4	1.7	0.7	7

Table 3 % Of similarity between ARISA profiles of biofilms from the same microcosm experiment as determined by the Jaccard index

% Of similarity	Cd experiment		Ni experiment		Zn experiment	
	Bacteria (%)	Eukaryotes (%)	Bacteria (%)	Eukaryotes (%)	Bacteria (%)	Eukaryotes (%)
Control/low	28.6	7.7	42.9	57.1	37.5	42.9
Control/high	42.9	25.0	0.0	0.0	25.0	22.2
Low/high	40.0	25.0	16.7	0.0	28.6	30.0

In bold letters, the highest % of similarity is shown in each experiment for either bacterial or eukaryotic communities

Percentages of similarity calculated using the Jaccard index (Table 3) were higher between the control and low metal treatment biofilms for both bacterial and eukaryotic communities in the Ni and Zn experiments, whereas the control and high metal treatment biofilms had the highest % of similarity in the Cd experiment (bacterial and eukaryotic communities).

Metal tolerance measurements with the short-term toxicity test

Dose/effect curves of biofilms from the microcosms are given in Fig. 1. Table 4 summarizes the values of EC_{50} and the maximum percentages of inhibition of β -glucosidase activity calculated from the dose/effect curves. In the Cd experiment, the increase of the normalized EC_{50} value was significant for both metal treatments (high and

low), when compared to the control biofilm. In the Ni experiment, a significant decrease of the normalized EC_{50} value was observed for the low metal treatment, and the normalized EC_{50} value increased markedly for the high metal treatment. In the Zn experiment, the increase of community tolerance to both Cd and Zn was significant for the high metal treatment, and tolerance levels of both the control and low metal treatments were similar.

A small but significant decrease of the maximum % inhibition levels was observed for both Cd-exposed biofilms. In the Ni experiment, maximum % inhibition levels also decreased for the Ni-exposed biofilms but not significantly (confidence intervals overlap). Maximum % inhibition levels decreased drastically for the high metal treatment in the Zn experiment with both Zn and Cd, and remained unchanged for the low metal treatment.

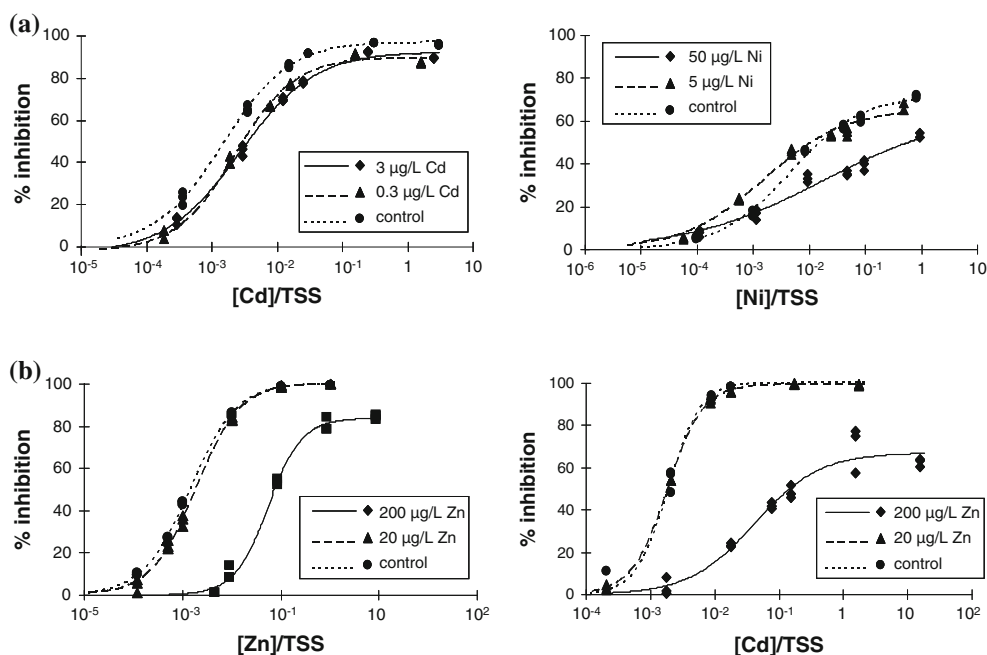


Fig. 1 Dose/effect curves from the short-term toxicity tests performed on control, low metal and high metal treatment biofilms for the Cd, Ni (a), and Zn experiments (b: both Zn and Cd were tested in

the Zn experiment). For each metal concentration tested, % inhibition of β -glucosidase activity are shown as triplicates. TSS-normalized metal concentrations are expressed as $g_{\text{metal}}/g_{\text{TSS}}$

Table 4 Normalized EC₅₀ values and maximum inhibition levels of β -glucosidase activity calculated from the dose/effect curves, with their 95% confidence intervals

Long-term metal exposure	Metal treatment	Short-term toxicity tests	Normalized EC ₅₀ in mg _{metal} /g _{TSS} (95% confidence interval)	% Maximum inhibition level (95% confidence interval)
Cd	Control	Cd	1.17 (0.85–1.49)	97 (96.2–97.9)
	Low metal		1.93 (1.56–2.30)	90.2 (88.2–92.2)
	High metal		2.21 (1.64–2.78)	92.3 (91.3–94.7)
Ni	Control	Ni	4.75 (3.35–6.15)	68 (65.2–71.2)
	Low metal		1.45 (0.74–2.15)	62.2 (59.0–66.8)
	High metal		19.21 (6.4–128.07)	63.2 (53.8–84.7)
Zn	Control	Zn	1.32 (1.15–1.49)	100.2 (99.2–101.1)
	Low metal		1.69 (1.20–2.18)	100.6 (99.4–102.2)
	High metal		46.26 (36.96–55.56)	84.8 (83.5–86.6)
	Control	Cd	1.56 (1.32–1.80)	100.4 (99.3–101.8)
	Low metal		1.50 (1.36–1.64)	99.1 (98.7–99.5)
	High metal		37.60 (16.24–58.97)	67.4 (62.3–74.3)

Discussion

Community tolerance

The β -glucosidase toxicity test proved to be an efficient tool to detect metal-induced tolerance of the heterotrophic component of biofilms, as a significant increase of the normalized EC₅₀ values was observed at concentrations of 0.3 $\mu\text{g/l}$ for Cd (and 3 $\mu\text{g/l}$ Cd), 50 $\mu\text{g/l}$ for Ni, and 200 $\mu\text{g/l}$ for Zn when compared to the corresponding control biofilms. Therefore metal-induced tolerance of the heterotrophic component of periphyton can be detected using the β -glucosidase toxicity test.

Another result is the impossibility to completely inhibit the heterotrophic activity of the metal-exposed biofilms even at high metal concentrations. Occurrence of such a maximum inhibition level (inferior to 100%) with the β -glucosidase toxicity test had already been observed in former experiments on natural periphyton from a metal-contaminated urban area (Fechner et al. 2010a). Several hypotheses can be proposed to explain the presence of a maximum level of inhibition. The first hypothesis is that exposure is not long enough to inhibit β -glucosidase activity completely. This hypothesis was tested and invalidated by increasing exposure time with Ni from 1 to 2 and 4 h (unpublished data). The maximum inhibition level remained unchanged for the three exposure times tested. Even though this hypothesis has only been tested with Ni, we can assume that there is another explanation to the presence of a maximum level of inhibition lower than 100%. The second hypothesis is the presence of a stock of exoenzymes (not bound to the cells) in the biofilm matrix (Romaní et al. 2008), which would be responsible for the residual fluorescence signal at high concentrations of metal.

The production of EPS (mucilage) is a well-known mechanism of protection from toxicants (Sabater et al. 2007). Therefore the protective effect of the biofilm matrix is also part of the biological response of biofilms to metal exposure that we want to evaluate using this short-term toxicity test. The third hypothesis is the presence of metal-resistant species that still have a β -glucosidase activity even at high concentrations of metal. Testing this hypothesis would require further investigation to detect metal-resistant species, which was beyond the scope of the present study.

Although the presence of a maximum level of inhibition below 100% integrates complex biological mechanisms, it reflects biofilms' sensitivity to metals. In the present study, maximum inhibition levels decreased as community tolerance increased. Therefore a maximum level of inhibition below 100% indicates metal-induced modifications of community structure as well as the joint increase of community tolerance (measured as an increase in EC₅₀ value).

Community descriptors

The modifications of general biofilm parameters (dry weight, algal biomass and heterotrophic activity) globally reflects the effects of metal exposure, but does not always totally coincide with PICT measurements. Indeed, joint effects of metal exposure were observed on both tolerance levels and periphyton biomass parameters for the high metal treatments in the Cd and Zn experiments. According to Blanck et al. (1988) and Blanck (2002), a relationship between induced tolerance and changes in community structure should preferably be obtained to conclude on a PICT effect. Biomass descriptors are rough indicators of community structure that have been commonly used as descriptors of biofilm biomass and may sometimes express

the long-term effects of toxicants or water chemistry on periphytic communities (Burns and Ryder 2001; Sabater et al. 2007). Yet they do not necessarily allow detection of subtle variations in community structure. It is therefore most interesting to relate tolerance acquisition to modifications of fingerprints which give indications on modifications of community structure.

ARISA fingerprints are most useful for enlightening changes in the structures of both bacterial and eukaryotic communities with metal exposure. ARISA fingerprints in this study revealed a decrease of bacterial diversity in all experiments, with distinct effects for each exposure level. They also revealed that eukaryotes were affected by metal exposure, but not necessarily in terms of a decline of community diversity as observed for bacterial communities. Increased eukaryotic diversity of metal-exposed periphyton has already been observed: for instance, in the study by Serra et al. (2009), control communities were dominated by a pioneer diatom species which was affected by Cu exposure, which thus lead to an increase of diversity in the Cu-exposed communities.

In some cases, for instance for the low metal treatment biofilms in the Ni and Zn experiments, ARISA profiles revealed changes in community structure whereas no increase of tolerance (expressed as normalized EC_{50}) could be detected on those communities. ARISA therefore provides a complementary approach to the detection of tolerance acquisition with a PICT methodology. It indeed allows the detection of metal-induced modifications of community structure that are not necessarily accompanied by an increase of tolerance. Moreover, ARISA provides insight into modifications of community structure that are not necessarily detected using classical biomass descriptors as can be observed for Ni-exposed biofilms for which variations of both DW and Chl-*a* are low whereas the Jaccard and Shannon indexes reveal deep changes in both bacterial and eukaryotic communities.

Co-tolerance and implication for the use of the PICT method under multi-metallic exposure

This study shows that co-tolerance mechanisms affect the heterotrophic component of biofilms, and can be detected using the β -glucosidase toxicity test. Occurrence of co-tolerance or multi-tolerance is common for both algal and bacterial species (Gustavson and Wängberg 1995; Paulsson et al. 2000). Soldo and Behra (2000) have observed co-tolerance to Ni, Ag and Zn in Cu-exposed biofilms using a photosynthetic toxicity test. Co-tolerance can be related to diverse mechanisms (Gustavson et al. 1999), for instance mucilage production, which is a non-specific defense means, and thus likely to affect tolerance to several metals (Soldo and Behra 2000).

As co-tolerance means that a community exposed to a given toxic agent becomes tolerant to another one, proper identification of the exact selection pressure might become more difficult when it occurs (Blanck et al. 1988, 2009). Application of the same methodology to natural in situ samples exposed to complex multi-metallic contaminations that are typical of urban areas is thus likely to lead to complicated tolerance patterns. In field studies, biofilm metal tolerance levels could therefore be considered as an early-warning response to a global multi-metallic urban contamination, without trying to affect a given metal tolerance level to exposure to that same exact metal.

Towards field evaluation of PICT effects with the β -glucosidase toxicity test

In the present study, metal exposure levels were close to realistic exposure levels observed in common urban rivers. In particular, in each experiment, the low metal treatment was within the range of observed total metal concentrations in typical urban rivers (Bourgeault et al. 2010; Tusseau-Vuillemin et al. 2007) and the high metal treatment was chosen one order of magnitude higher. However, in most studies, metal exposure levels used for periphyton grown in microcosms (or other aquaria systems) usually tend to be higher. For instance, Ivorra et al. (2000, 2002) exposed biofilms to Zn concentrations of 1000–3000 $\mu\text{g/l}$ and Cd concentrations of 64–100 $\mu\text{g/l}$, and Morin et al. (2008) exposed biofilms to Cd concentrations of 10 and 100 $\mu\text{g/l}$.

PICT effects are not always easily detected in indoor aquaria experiments at low metal concentrations and with realistic exposure lengths: Paulsson et al. (2000) exposed periphyton to Zn from 3 $\mu\text{g/l}$ to 2 mg/l and observed an increase of tolerance at exposure concentrations higher than 600 $\mu\text{g/l}$. PICT sometimes even prove to be undetectable for periphyton, for instance after a 20 days exposure to Cu at concentrations varying from 1 to 15 $\mu\text{g/l}$, although an increase of tolerance could be detected for phytoplankton exposed simultaneously (Gustavson and Wängberg 1995). Similarly, increases of metal tolerance of natural communities collected in contaminated areas are mostly reported for sites with relatively high contamination levels, usually due to mining activities: Admiraal et al. (1999) detected an increase of Zn tolerance of bacterial communities collected at sites with Zn contamination levels above 200 $\mu\text{g/l}$ and Lehmann et al. (1999) observed an increase of Zn community tolerance of periphyton collected at sites with Zn contamination levels above 60 $\mu\text{g/l}$.

In the last two examples, the authors also report difficulties in measuring Zn tolerance of algal communities with short-term toxicity tests based on the inhibition of ^{14}C -incorporation in microalgae (Admiraal et al. 1999; Lehmann et al. 1999). Metal-induced increases of

periphyton tolerance have already been described in several studies, usually with Cu and short-term toxicity tests using photosynthetic activity as an endpoint, as Cu alters the algal component of these communities (see for instance Serra et al. 2010; Soldo and Behra 2000). Although metals like Cd, Zn and Ni are certainly toxic to autotrophs, they do not specifically impair photosynthesis compared to a metal like Cu, which is a well-known inhibitor of the PSII-photosystem (Maksymiec 1997). It might therefore be more difficult to detect tolerance acquisition to metals like Cd, Zn and Ni using photosynthesis as the endpoint in toxicity tests, as observed by Lehmann et al. (1999). The toxicity test used in the present study, which allows to measure the metal tolerance of the heterotrophic component of periphyton, appears to be sensitive to low levels of exposure. Indeed, subtle metal-induced community changes at low and realistic exposure levels were successfully detected with a simple experimental setup. The toxicity test used to assess heterotrophic community tolerance combined with the ARISA technique is therefore appropriate to detect the biological response of biofilms exposed to different metals at environmental contamination levels.

Biofilm inoculum was collected in a small river in the Seine river basin (Rémarde river). It is obvious that laboratory-grown biofilms are different from the original periphyton collected in situ. In particular, laboratory biofilms are usually much less complex communities as observed by Serra et al. (2009), and confirmed in this study by comparison of Shannon diversity indexes from the Rémarde biofilms and the microcosms' biofilms. Therefore, more subtle or even different biological responses might be expected in field studies using the same PICT methodology on in situ-exposed periphyton (for instance tolerance acquisition of the heterotrophic communities might be observed at lower exposure levels than in the present study). The present study was indeed designed to validate the use of the β -glucosidase toxicity test to assess tolerance acquisition of periphyton exposed to environmental metal concentrations. These microcosm experiments showed that the PICT methodology developed allows to detect tolerance acquisition at realistic exposure levels, which means it can be further used in field studies.

Conclusion

The aim of this study was to find out whether the short-term toxicity test based on a measure of heterotrophic activity was sensitive enough to detect community tolerance acquisition on periphyton under realistic metal exposure. Tolerance acquisition was indeed measured for Cd, Ni and Zn with the β -glucosidase toxicity test under controlled conditions at low exposure levels. With Cd and

Zn, maximum % inhibition levels inferior to 100% appeared jointly with the increase of EC₅₀ values. With Ni, maximum % inhibition level, which was lower than 100% for the control, further decreased with metal exposure. These results indicate that both the increase of EC₅₀ values and the decrease of the maximum inhibition levels can be used to assess the biological response of periphyton to metal exposure. Bacterial and eukaryotic ARISA profiles confirmed that periphyton communities were impacted by metal exposure. In particular, a decrease of bacterial diversity was observed on fingerprints corresponding to metal-exposed biofilms. Combining the β -glucosidase toxicity test with periphyton biomass descriptors and the ARISA fingerprinting technique will thus help to examine the complex biological response of natural biofilms exposed to subtle urban multi-contamination in situ.

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