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TECHNICAL NOTE

# Adapting an enzymatic toxicity test to allow comparative evaluation of natural freshwater biofilms' tolerance to metals

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**Abstract** A simple, low-cost and non-radioactive short-term toxicity test was developed to study the effects of urban metals on natural freshwater periphytic communities. $\beta$-glucosidase activity of natural freshwater biofilms collected in situ was chosen as an endpoint. Metals (Cd, Cu, Ni, Pb, and Zn) successfully inhibited bacterial enzymatic activity after a 1-h exposure enabling the calculation of $EC_{50}$. The $EC_{50}$ value of a biofilm sample varied with the Total Suspended Solids concentration (TSS) of the biofilm suspension, showing that $EC_{50}$ values (expressed as total added metal concentrations) are not representative of the bioavailable metal concentration during the toxicity test. For Cu, Cd, Ni, Zn and Pb, the $EC_{50}$ values increased linearly with the TSS concentration leading us to define a normalized $EC_{50}$: the value of the $EC_{50}$ divided by the corresponding TSS concentration. Normalized $EC_{50}$ proved to be a robust, reliable way to assess metal tolerance of a biofilm for Cd, Cu, Ni, Zn and Pb. Normalized $EC_{50}$ obtained, expressed as $kg_{metal}/g_{TSS}$, varied between 0.2 to 7.6 for Cu, 1 to 8 for Cd, 1.8 to 92.3 for Ni, 1.8 to 76.6 for Zn and 25 to 189 for Pb.

**Keywords** Periphyton · Enzymatic activity · Short-term toxicity test · Metal tolerance

## Introduction

Anthropogenic pressure in aquatic systems leads to the presence of a mixture of toxicants in a range of concentrations that are usually not lethal but that are the cause of more subtle, chronic effects on the composition and behaviour of biological communities. It is not easy to establish the link between long-term exposure to multiple and diffuse contaminations and a possible biological response in natural ecosystems. Indeed the biological response is particularly complex because of the exposed organisms' adaptation, the lengths of time that are required to observe chronic effects and the multiplicity of stress parameters both natural and anthropogenic.

In this context, it is necessary to develop methods allowing to discriminate pollution-induced modifications from those due to other causes. Exposure to a toxicant induces community changes by selecting tolerant components, which can be either species, genotypes or phenotypes (Bérard et al. 2002; Blanck 2002). As a result, the exposed community becomes less sensitive to the toxicant. Community tolerance, as described in the PICT—Pollution Induced Community Tolerance—concept (Blanck 2002), is thus an interesting tool to study the effects of a chronic contamination.

The determination of community tolerance to a given contaminant is achieved by the means of short-term toxicity tests. Community tolerance, usually expressed as an Effect Concentration (EC), is a complex parameter which integrates the response of numerous species within the studied community and their interactions. Comparisons of tolerance levels (usually $EC_{50}$) allow to determine if the toxicant has affected the studied community. The toxicity tests are performed using metabolic endpoints such as bacterial or photosynthetic activities, mostly with the use of radioactive reagents (Blanck 2002; Blanck et al. 2003).

Periphyton is a biofilm composed of algae, bacteria, fungi, protozoan and other species that develop on submerged objects such as rocks, macrophytes and other

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substrates. It plays an important part in aquatic ecosystems as primary producer and can be useful in assessing the overall health of an aquatic system. For instance, biofilms are regarded as good early warning indicators of the presence of toxicants in aquatic ecosystems (Sabater et al. 2007). They have also been used for PICT measurements with herbicides, antifouling agents or metals (Blanck 2002; Sabater et al. 2007).

Metal toxicity has long been studied on aquatic organisms (Tessier and Turner 1995). As regards micro-organisms and in particular bacteria, many toxicity tests have been widely used, for instance the classical Microtox test (Bulich and Isenberg 1981). However, those tests do not provide information on the impact of metal exposure on natural organisms. Moreover, toxicity testing at the community level provides a much broader context for the assessment of environmental contamination than studies on individual species (Clements and Rohr 2009). It is therefore most interesting to develop toxicity tests to study the effects of metals on natural biofilms.

Exo-enzymes, and in particular $\beta$-glucosidase, which controls essential metabolic pathways in aquatic ecosystems, are interesting to study the effects of environmental perturbation on the functioning of aquatic ecosystems (Admiraal and Tubbing 1991). Exo-enzymatic activities have already been used for metal toxicity testing on bacterioplankton (Tubbing and Admiraal 1991). $\beta$-glucosidase activity, which represents the global activity of heterotrophic micro-organisms (Chrost 1991), is therefore an interesting endpoint for a short-term toxicity test used to assess metal tolerance of natural biofilms. To avoid problems related to biofilm thickness such as poor diffusion of the substrate inside the biofilm matrix and also to obtain comparable activity measurements via better homogenization of the biofilms, $\beta$-glucosidase activity is usually measured on disrupted biofilms (biofilm suspensions) (Romaní et al. 2008; Smucker et al. 2009).

The issue of bioavailability in the medium during the short-term toxicity tests is not often addressed. It is however an essential point when quantifying community tolerance with $EC_{50}$ values. $EC_{50}$ are usually expressed as nominal (or total added) metal concentrations and are not good indicators of metal exposure: if bioavailability is low during the test, it can be erroneously interpreted as an increase in community tolerance (Blanck 2002; Blanck et al. 2003). Bioavailability can be affected either by the test media which is usually filtered river water, a problem which can be eliminated by using the same test medium for all biofilm samples (Blanck et al. 2003), or by the periphyton composition itself. Indeed periphyton is a complex matrix of micro-organisms, particulate matter and extracellular polymers (EPS) (Wimpenny 2000). The composition of the biofilm matrix is influenced by its environment, for instance via the production of EPS (Serra and Guasch 2009) or cellular ligands (Soldo and Behra 2000), or the presence of iron and manganese oxides, which can sometimes induce precipitation of metals within biofilms (Admiraal et al. 1999; Lehmann et al. 1999): all these elements are likely to modify metal speciation during metal toxicity testing. Indeed the biofilm composition affects metal bioavailability and thus metal exposure levels during the tests. For instance, the presence of inorganic particles like silt in natural river biofilms can influence metal bioavailability and thus modify the $EC_{50}$ value obtained with a short-term toxicity test.

This paper presents the development of a new method allowing comparative evaluation of natural freshwater biofilms' tolerance to metals using exo-enzymatic activities. We chose to focus on several metals present in urban rivers (Cd, Cu, Ni, Pb and Zn) and on their effects on the $\beta$-glucosidase activity of the heterotrophic component of periphyton. In order to obtain comparable tolerance levels, we also investigated the influence of the biofilm concentration on toxicity tests' results: for each metal, $EC_{50}$ values were calculated for several dilutions of the same biofilm suspension. We then developed a simple normalization process to take into account metal bioavailability during the toxicity tests.

## Materials and methods

### Sampling sites

Periphyton was collected on Low Density PolyEthylene plastic membranes that were vertically attached and immersed either in the Seine river at Andresy (downstream from Paris, Strahler: 8, mean annual flow in 2007: 450 $m^3/s$) or the Marne river at Saint-Maurice (upstream from Paris, Strahler: 6; mean annual flow in 2007: 112 $m^3/s$). Andresy is located approximately 4 kilometres downstream the effluent discharge from the sewer treatment plant Seine-Aval (nominal capacity $8 \times 10^6$ inhabitants, flow 24 $m^3/s$). Periphyton was collected on plastic membranes all year round after colonization times of at least 7 days. Biofilms were collected from both sites at different periods of the year and with different colonization times to adjust the methodology of the toxicity test.

### Preparation of the periphyton suspension

Colonized membranes were taken out of the water and carried in 250 ml glass bottles filled with mineral water (Grand Barbier, Mont-Dore, France). Blanck et al. (2002, 2003) recommend to use the same medium for all toxicity tests, in order to avoid changes in metal bioavailability

during metal toxicity testing. Mineral water was used instead of a buffer, in order to avoid modifying metal speciation within the biofilm suspension during the toxicity tests by adding metal complexing agents. Mont-Dore water, which is weakly mineralized, was chosen to avoid adding too many mineral substances during the experiments (composition in mg/l: $Ca^{2+}$: 4.1, $Mg^{2+}$: 1.7, $Na^{+}$: 2.7, $K^{+}$:0.9, $SO_4^{2-}$: 1.1, $HCO_3^{-}$: 25.8, $Cl^{-}$:0.9, $NO_3^{-}$: 0.8, $NO_2$: 0.0). The bottles were taken back to the laboratory in a cooler and then rinsed with mineral water. The membranes were hand-scraped to remove periphyton. The periphyton suspensions (made with mineral water) were homogenized by fast magnetic stirring before being aliquoted for periphyton analyses or toxicity testing.

### Characterization of the periphyton suspensions

Periphyton samples were characterized by their Dry Weight (DW) expressed in gram per $m^2$ of plastic membrane. DW were determined by filtration of a known volume of biofilm suspension (between 5 to 20 ml according to the concentration of the suspension) on calcinated 0.7 µm-glass-fiber filters (GF/F Whatmann®, Kent, UK) further dried out at 100°C for 2–3 h.

Periphyton suspensions used for the toxicity tests were characterized by their TSS (Total Suspended Solids) concentrations expressed in gram of dry matter per liter of biofilm suspension. TSS concentrations were calculated simultaneously with DW concentrations by filtration of a known volume of suspension. TSS concentrations were measured for each dilution of periphyton suspensions used for the toxicity tests.

### β-glucosidase activity measurement

The enzymatic activity of periphyton was determined using methylumbelliferone-β-D-glucopyranoside or MUF-GLU (Sigma-Aldrich). Prior to its addition to the periphyton samples, MUF-GLU was dissolved in demineralized water in order to achieve 4 mM MUF-GLU. The MUF-GLU stock solution was stored at a deep-freeze temperature of −20°C. Aliquots of this stock solution (100 or 200 µl) were added to 4 ml of periphyton suspension which was vortexed and then left to incubate in the dark on an orbital shaker for 30 min at 20°C. 400 µl of 1 M NaOH were then added to stop the enzymatic reaction according to Li and Chrost (2006). The assays were performed at saturating levels of substrate (100 or 200 µM MUF-GLU) after determination of the saturation concentration. Fluorescence of MUF was measured without delay following centrifugation (15 min, 3000 rpm) by adding about 3 ml of the supernatant to a UV-cuvette and using a SAFAS SP 2000 Xenius spectrofluorimeter at an excitation wavelength of 348 nm and an emission wavelength of 448 nm. For each periphyton assay, a standard curve was also produced by plotting the peak heights of a range of MUF solutions as a function of the corresponding MUF concentrations. Results showed that fluorescence peak height was proportional to the MUF concentration. Therefore β-glucosidase activity was expressed as MUF fluorescence peak height at 448 nm. Control samples were assessed with a null incubation time by adding NaOH directly after the substrate. Fluorescence of the control was always negligible when compared to fluorescence levels of other samples and pH was >13 in all supernatants due to NaOH addition.

### β-glucosidase toxicity test on periphyton

Solutions of metals were prepared using demineralized water and metallic salts $Cd(NO_3)_2$, $ZnSO_4(H_2O)_7$, $CuCl_2$, and $Pb(NO_3)_2$ (Acros Organics, Geel, Belgium).

3.96 ml of periphyton suspension in mineral water were added to 15 ml plastic tubes with 40 µl of metal solution. One control (40 µl of demineralized water instead of metal solution) and 5–6 metal concentrations were tested in triplicates. Nominal exposure concentrations of metals varied between 0.32 and 63.5 mg/l for Cu, 0.25 and 100 mg/l for Cd, 0.1 and 599 mg/l for Ni, 0.1 and 657.7 mg/l for Zn, 0.3 to 2084 mg/l for Pb. Concentrations were chosen so that maximum inhibition of β-glucosidase activity was reached. For that purpose the highest concentration tested in the range was often at least ten times higher than the second highest concentration tested. The tubes were vortexed and left to incubate in the dark at 20°C on an orbital shaker for 1 h. A 1-h exposure time was sufficient to inhibit β-glucosidase activity in all experiments. β-glucosidase activity was then measured according to the protocol described above. Preliminary studies showed that fluorescence levels were not disrupted by adding high concentrations of metal. Metal effect on β-glucosidase activity was reported as the decrease of MUF fluorescence signal. Dose/effect curves were obtained by plotting MUF fluorescence signal as a function of metal concentration. The curves were fitted to the Hill model (Hill 1910) by non-linear regression using the algorithm of Marquardt (1963) and a non-linear regression program proposed by Duggleby (1981) (for more details, see http://eric.vindimian.9online.fr). The general form of the curve fitted on the data is:

$$F(x) = (p_{\max} - p_0)\frac{x^H}{x^H + \mathrm{EC}_{50}^H} + p_0$$

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 $F(x)$ is a probability

law. H is the Hill number (slope of the toxicity curve) and $EC_{50}$ the median effect concentration. Each parameter ($p_0$, $p_{max}$, $EC_{50}$ and H) is adjusted for the calculation. Confidence intervals around the fitted parameters were estimated using a Bootstrap method. Dose/effect curves are represented as an augmentation of the percentage of inhibition of the MUF fluorescence signal of the control with the metal concentration.

### Influence of the periphyton suspension concentration

For each metal, dilution experiments (two per metal, see Tables 1 and 3) were performed to investigate the influence of biofilm suspensions' concentrations on the measurement of tolerance: for each dilution experiment, three dilutions were prepared in mineral water from a unique biofilm suspension and used to perform three toxicity tests. Metals were tested separately i.e. on different biofilms (collected at different times of the year): testing different biofilms allowed to validate the fact that the influence of the biofilm suspension's concentration on tolerance measurement is not related to the particular composition of a given biofilm sample but to modifications of metal bioavailability during the toxicity tests. Dilution factors ranged between 2 and 10. The three dilutions were used to perform $\beta$-glucosidase toxicity tests and determine three $EC_{50}$ values. Data from the three toxicity tests were also gathered on the same plot to produce a unique dose/effect curve with the % inhibition of $\beta$-glucosidase activity as a function of TSS-normalized metal concentrations from the three toxicity tests.

**Table 2** $EC_{50}$ (mg/l) and normalized $EC_{50}$ ($*10^3$ $g_{metal}/g_{TSS}$) obtained for Cu, Cd, Ni, Zn and Pb from biofilms collected at Saint-Maurice and Andresy (a) with their 95% confidence intervals (in brackets)

| Metal | $EC_{50}$ | $EC_{50}$/TSS |
|---|---|---|
| Cu | 12.15[a] (11.15–13.25) | 7.63 (7.01–8.34) |
| | 1.24[a] (0.86–2.82) | 1.81 (0.00–4.52) |
| | 0.54 (0.46–0.63) | 0.33 (0.28–0.38) |
| | 0.18 (0.17–0.19) | 0.36 (0.29–0.44) |
| Cd | 12.78[a] (5.92–25.15) | 8.03 (0.23–15.84) |
| | 2.49 (2.33–2.64) | 2.54 (2.38–2.69) |
| Ni | 25.24[a] (12.78–778.06) | 92.26 (48.22–2936.09) |
| | 43.40[a] (38.32–49.13) | 28.18 (23.37–32.99) |
| | 7.40 (7.09–7.77) | 7.54 (7.22–7.92) |
| Zn | 19.37[a] (14.87–32.91) | 76.58 (41.28–111.88) |
| | 0.68 (0.54–0.85) | 1.38 (0.94–1.81) |
| Pb | 50.13[a] (36.00–51.63) | 189.18 (135.88–194.81) |
| | 47.16 (39.70–55.10) | 34.28 (28.4–40.1) |

Normalized values are multiplied by $10^3$ for more convenient reading

## Results and discussion

### $\beta$-glucosidase inhibition by metals

Table 1 gives information on the sampled biofilms (sampling site, sampling date, colonization time, DW and metal tested). All the metals that were tested (Cd, Cu, Ni, Pb, Zn) successfully inhibited the $\beta$-glucosidase activity of periphyton after a 1-h exposure and $EC_{50}$ could be assessed

**Table 1** TSS, colonization time and metals tested on biofilms collected at Saint-Maurice and Andresy

| Sampling site | Sampling date | Colonization time (days) | DW ($g/m^2$) | Metal tested |
|---|---|---|---|---|
| Saint-Maurice | 9 Jan 2008 | 44 | 7.51 | Cu (d) |
| | 14 Jan 2008 | 49 | 8.72 | Cu, Cd (d) |
| | 21 Jan 2008 | 56 | 6.89 | Cd (d) |
| | 28 Jan 2008 | 54 | 30.04 | Cd (d) |
| | 18 Feb 2008 | 75 | 102.88 | Cu (d) |
| | 25 Feb 2008 | 83 | 140.45 | Ni (d) |
| | 11 Mar 2008 | 98 | 51.32 | Pb |
| | 18 Mar 2008 | 105 | 57.95 | Zn (d) |
| | 25 Mar 2008 | 50 | 21.38 | Pb (d) |
| | 31 Mar 2008 | 56 | 21.82 | Ni (d) |
| | 07 Apr 2008 | 63 | 30.58 | Zn (d) |
| | 01 Jul 2008 | 69 | 54.35 | Pb (d) |
| | 07 Jul 2008 | 76 | 85.78 | Cu, Cd, Zn, Ni |
| Andresy | 06 Nov 2006 | 12 | 12.72 | Cu, Cd, Zn |
| | 14 Nov 2006 | 7 | 2.12 | Ni, Pb |
| | 28 Nov 2006 | 15 | 15.40 | Ni |
| | 11 Dec 2006 | 14 | 5.48 | Cu |

*nd* not determined, *d* a dilution experiment was performed

using the dose/effect curves. The $EC_{50}$ values that we obtained cover a large range of values (Tables 2 and 3). Such variations of tolerance of complex, natural communities are not surprising considering that susceptibility to toxicants can vary by several orders of magnitude among taxonomically different species (Slooff et al. 1983) and is a well known phenomenon in biofilms varying in age and succession status (Sabater et al. 2007). Interpretation of such variability is outside of the scope of the present study, which was to further develop an enzymatic toxicity test. The $EC_{50}$ values that we obtained are in the range of values from various studies using radioactive short-term toxicity tests, for instance with Cd (Lehmann et al. 1999) or Zn (Admiraal et al. 1999; Blanck et al. 2003; Lehmann et al. 1999) using thymidine incorporation as an endpoint. The $EC_{50}$ of the biofilms collected at Andresy are rather high and closer to $EC_{50}$ measured on sludge by inhibition of alanine-aminopeptidase activity (Dalzell et al. 2002).

**Table 3** $EC_{50}$ (mg/l) and normalized $EC_{50}$ ($*10^3$ $g_{metal}/g_{TSS}$), with their 95% confidence intervals (in brackets), for Cu, Cd, Ni, Zn and Pb from biofilms collected at Saint-Maurice used in the dilution experiments

| Metal | $EC_{50}$ | $EC_{50}$/TSS | Global $EC_{50}$/TSS from the unique dose effect curve (dilution experiments) |
|---|---|---|---|
| Cu | 0.44 (0.42–0.46) | 0.3 (0.27–0.32) | 0.32 (0.30–0.34) |
| | 0.25 (0.18–0.41) | 0.4 (0.11–0.69) | |
| | 0.07 (0.05–0.12) | 0.29 (0.10–0.48) | |
| | 0.87 (0.79–0.96) | 0.28 (0.21–0.35) | 0.24 (0.22–0.26) |
| | 0.36 (0.33–0.39) | 0.25 (0.22–0.28) | |
| | 0.1 (0.10–0.11) | 0.18 (0.14–0.22) | |
| Cd | 5.25 (4.15–7.49) | 3.25 (1.24–5.26) | 2.89 (1.84–4.36) |
| | 2.79 (1.71–7.28) | 3.97 (0–10.73) | |
| | 1.38 (1.15–10.69) | 2.43 (1.39–3.46) | |
| | 6.18 (5.49–7.14) | 5.49 (4.62–6.36) | 6.37 (5.72–7.16) |
| | 3.92 (3.58–4.34) | 7.37 (6.02–8.71) | |
| | 1.38 (1.15–1.69) | 6.96 (0–20.39) | |
| | 2.43 (2.17–2.73) | 1.03 (0.89–1.17) | 1.96 (1.48–2.56) |
| | 2 (1.72–2.34) | 2.68 (2.22–3.13) | |
| | 0.45 (0.34–0.59) | 1.19 (0.61–1.77) | |
| Ni | 3.57 (3.16–4.03) | 1.85 (1.59–2.11) | 2.55 (2.05–3.08) |
| | 1.67 (1.29–2.16) | 2.67 (1.71–3.63) | |
| | 1.36 (1.02–1.86) | 3.36 (0.54–6.19) | |
| | 44.02 (40.42–48.80) | 31.35 (16.91–45.78) | 27.32 (19.50-40.23) |
| | 18.24 (15.72–21.19) | 25.54 (11.42–39.66) | |
| | 9.67 (8.19–11.6) | 16.47 (7.11–25.83) | |
| Zn | 8.84 (7.92–9.70) | 1.90 (1.70–2.09) | 3.00 (2.59–3.52) |
| | 3.12 (2.79–3.47) | 2.36 (2.07–2.65) | |
| | 1.52 (1.28–1.77) | 3.87 (3.08–4.67) | |
| | 4.52 (4.07–4.99) | 2.12 (1.86–2.39) | 2.72 (2.45–2.99) |
| | 2.39 (2.22–2.56) | 2.32 (1.76–2.87) | |
| | 1.87 (1.66–2.11) | 3.55 (1.14–5.97) | |
| Pb | 57.28 (50.66–65.63) | 38.49 (32.33–44.65) | 62.25 (50.90–78.74) |
| | 77.57 (66.42–91.77) | 85.95 (67.99–103.91) | |
| | 47.5 (42.65–53.19) | 110.12 (66.78–153.46) | |
| | 51.96 (50.16–53.67) | 25.22 (22.40–28.04) | 31.50 (28.95–33.54) |
| | 32.4 (29.73–35.39) | 32.86 (29.82–35.90) | |
| | 24.9 (22.25–27.36) | 40.27 (31.76–48.78) | |

Normalized values are multiplied by $10^3$ for more convenient reading. For the dilution experiments, the global $EC_{50}$ value obtained from the unique dose/effect curve calculated with the TSS-normalized metal concentrations and corresponding inhibition data is shown (last column)

## Bioavailability of metals using the $\beta$-glucosidase toxicity test

The issue we want to address is that the $EC_{50}$ value for a given biofilm varies with the concentration of the biofilm suspension. Yet, in order to compare the tolerance of communities exposed to different levels of contamination—which is compulsory when trying to investigate pollution-induced community modifications—it is important to develop a measure of the short-term toxicity of the contaminant (expressed as an $EC_{50}$ value) that does not depend on metal bioavailability during the toxicity test.

To investigate this phenomenon, $\beta$-glucosidase toxicity tests were performed on dilution series of the same biofilm suspension (three dilutions of a biofilm suspension for each dilution experiment). Biofilm suspensions were not diluted more than 2–10 times so that there should be no radical changes in the composition of the community. The biofilm suspensions' concentrations were roughly estimated by their TSS concentrations. For each dilution experiment, $EC_{50}$ values were calculated using the three dose/effect curves corresponding to the three biofilm dilutions. Results show that the more concentrated the biofilm suspension, the greater the $EC_{50}$ value (Fig. 1; Table 3). Doubtlessly mineral or organic particles present in the biofilm suspension affect metal bioavailability to biofilm organisms. It all happens as if there was a physico-chemical equilibrium between the metal and the metal sorption sites (S), in which metal was partitioned between bioavailable and non-bioavailable metal (fixed to the sorption sites), which can be translated into Eqs. 1 and 2:

$$M_{\text{bioav}} + S \Leftrightarrow M_{\text{non-bioav}}$$

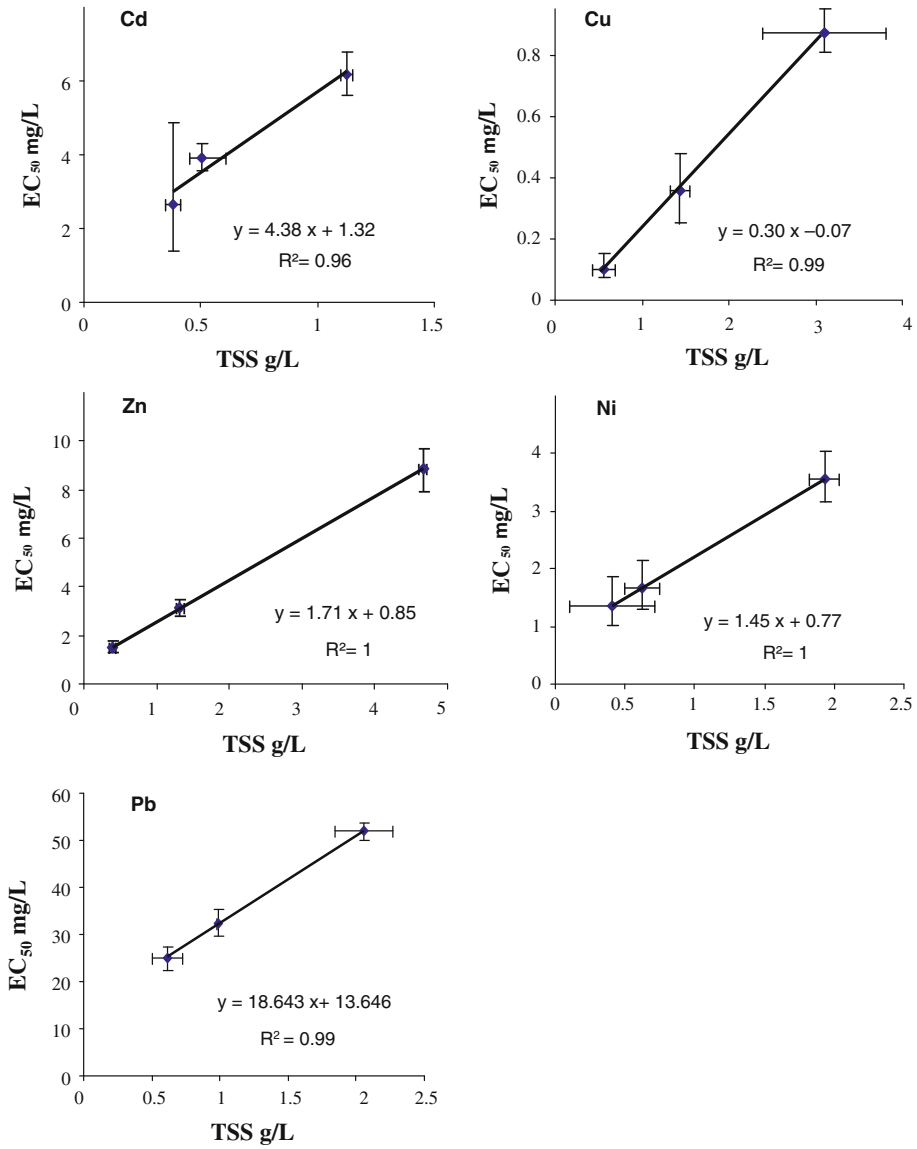


**Fig. 1** Effect of TSS (g/l) on $EC_{50}$ (mg/l) for $\beta$-glucosidase activity of biofilm with Cd (biofilm sampled on January 21st), Cu (biofilm sampled on February 18th), Zn (biofilm sampled on March 18th), Ni (biofilm sampled on February 25th) and Pb (biofilm sampled on July 1st). Linear regression was performed on the mean values of $EC_{50}$ and TSS concentrations. *Error bars* show 95% confidence intervals for $EC_{50}$ and standard deviations for TSS concentrations

$$K = \frac{[M_{\text{non-bioav}}]}{[M_{\text{bioav}}] \times [S]} \quad (1)$$

$$[M_{\text{tot}}] = [M_{\text{bioav}}] + [M_{\text{non-bioav}}] \quad (2)$$

where

| | |
|---|---|
| K | is the equilibrium constant |
| $M_{tot}$ | is the total metal concentration |
| $M_{bioav}$ | the bioavailable fraction of the metal |
| $M_{non\text{-}bioav}$ | the concentration of metal which is not bioavailable |

Equations 1 and 2 can be combined to express $M_{tot}$ as a function of [S] and $M_{bioav}$:

$$[M_{\text{tot}}] = [M_{\text{bioav}}] \times K \times [S] + [M_{\text{bioav}}] \quad (3)$$

Equation 3 can be written as Eq. 4 for a metal exposure level corresponding to the $EC_{50}$ value (expressed in total added metal concentrations):

$$EC_{50} = [M_{\text{bioav\_EC50}}] \times K \times [S] + [M_{\text{bioav\_EC50}}] \quad (4)$$

Plots of $EC_{50}$ values experimentally obtained for dilution series of a biofilm suspension and the corresponding TSS concentrations are shown in Fig. 1. If we further assume that the concentration of metal sorption sites [S] is proportional to the biofilm concentration ([TSS]), the linear relationship observed between the $EC_{50}$ and the corresponding TSS concentrations in Fig. 1 is validated by the theoretical Eq. 4. The *y*-intercept ($M_{bioav\_EC50}$) would be the hypothetical bioavailable metal concentration, at the $EC_{50}$, for a biofilm suspension with no particles ([S] = [TSS] = 0). It is therefore not a very realistic parameter to consider. As shown in Fig. 1, the *y*-intercept is also close to zero and cannot be estimated reliably by this method.

Variations of $EC_{50}$ values with the TSS concentrations led us to define a normalized $EC_{50}$, which is the value of the $EC_{50}$ (g/l) divided by the TSS concentration of the corresponding biofilm suspension (expressed in g/l). Figure 2 shows, for all five metals, on the one hand, the three distinct dose/effect curves corresponding to the three dilutions of a biofilm suspension (percentage of enzymatic activity inhibition as a function of metal concentration), and, on the other hand, the unique dose/effect curve obtained by gathering the data from the three toxicity tests: percentages of enzymatic activity inhibition from the three toxicity tests are expressed as a function of TSS-normalized concentrations of metal and plotted together. Therefore three distinct dose/effect curves can be merged into a single one by a simple normalization process.

Table 3 shows, for each dilution experiment, the three TSS-normalized $EC_{50}$ values, calculated from the three dose/effect curves from the three biofilm dilutions and the global $EC_{50}$ value calculated from the unique corresponding dose/effect curve. Considering the 95% confidence intervals, for one dilution experiment, the three TSS-normalized $EC_{50}$ values (from the three dilutions) can be considered equal for Cd, Cu, Ni and Zn. For some biofilm samples (Table 3), the 95% confidence intervals of the global $EC_{50}$ and the first biofilm dilution do not strictly overlap, but the limits of both confidence intervals remain close.

Modification of metal bioavailability, in the presence of environmental samples, during toxicity tests, is a problem likely to be encountered in any toxicity test, even when undisrupted biofilm is used, e.g. with colonized glass disks (as used for instance in Blanck et al. 2003; Lehmann et al. 1999). For instance, it is possible that Dissolved Organic Matter (DOM) or other abiotic components (e.g. silt particles) embedded in the biofilm matrix modify metal bioavailability during the toxicity tests but these effects are not easily detected. Indeed, in our study, it was possible to highlight problems related to metal bioavailability with toxicity tests performed on several dilutions of a biofilm suspension. Soldo and Behra (2000) already underlined the importance of metal speciation in the design of toxicity tests: they also used periphyton suspensions and normalized their toxicity data to the concentration of chlorophyll *a* in their periphyton suspensions.

It is obvious that the model we propose is a rough description of the phenomenon and that sorbed-metal is not limited to metal bound to particles. For instance, it is likely that DOM also plays a protective role on the biofilm micro-organisms in the biofilm suspensions as has previously been shown (Campbell 1995; Morel 1983)—more recently such a protective role of anthropic DOM from filtered wastewater samples on *Daphnia magna* was also uncovered (Buzier et al. 2006). However in complex, heavily-loaded biofilm suspensions, it is likely that the metal/particles equilibrium is predominant. In our model, the TSS concentration provides a rough estimate of a quantity of matter (mineral and organic) which acts directly on metal bioavailability without presuming on the nature of the matter considered (silt particles, EPS—produced by microorganisms, DOM, etc.). With TSS-normalization, the variation of metal tolerance ($EC_{50}$) with the dilution of the biofilm suspension disappears. Consequently, a normalized $EC_{50}$ is a reliable and robust estimation of metal tolerance that does not depend on experimental conditions as standard $EC_{50}$ do: it can thus be used to compare different biofilms which was beyond the scope of this study.

### *β*-glucosidase toxicity tests with Pb

As far as Pb is concerned, the relationship between the concentration of the biofilm suspension and the toxicity of the metal is less clear (Table 3). Indeed metal

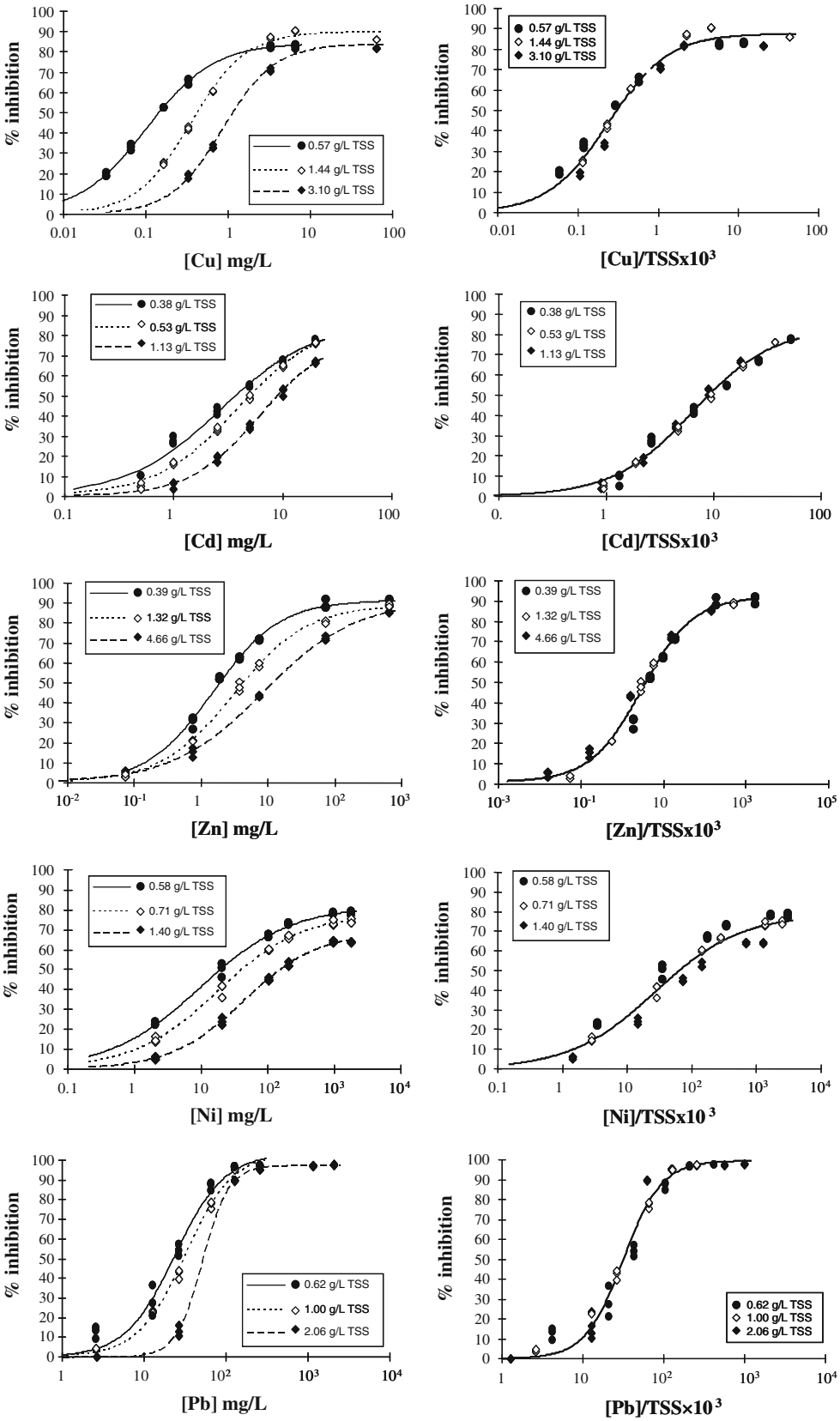


**Fig. 2** Concentration/effect curves for three dilutions of a biofilm suspension with % inhibition of $\beta$-glucosidase activity of biofilm as a function of metal concentrations (*left graph*) or normalized metal concentrations (*right graph*) for Cu, Cd, Zn, Ni and Pb

bioavailability and toxicity for Pb are likely to be affected differently by interactions with the particles in the biofilm suspension than for the other four metals studied. The difference of behaviour between Pb and the other metals that were tested in this study has been noticed before in the presence of metal ligands: for instance, both Tsiridis et al. (2006) and Sánchez-Marin et al. (2007) observed an increase of Pb toxicity in the presence of humic acids. Possible explanations mentioned in those studies are modifications of cell surface charge by DOM adsorption to cell membranes and the formation of ternary complexes between Pb, DOM components and the cell membrane, which could explain the increased uptake of Pb by organisms in the presence of DOM (Slaveykova 2007). Depending on its composition, DOM can also have a protective effect against Pb toxicity (Sánchez-Marín et al. 2010). It is therefore difficult to model Pb toxicity to aquatic organisms as a function of Pb speciation. In our study, Pb bioavailability is probably affected differently by particles or DOM in the biofilm suspensions, as the biofilms' composition varied between both dilution experiments. Hence normalization of $EC_{50}$ might not be a sufficient means to reliably assess lead tolerance of a heterotrophic periphyton community.

## Conclusion

This study was a first step towards assessing metal tolerance of freshwater biofilms. It focused on the development and interpretation of a short-term toxicity test based on the measure of the $\beta$-glucosidase activity of the heterotrophic part in biofilms. The test was developed on natural biofilm samples collected in situ at two different sites, and at different dates. Our first results show that $\beta$-glucosidase activity is inhibited by all the metals tested, and that $EC_{50}$ values can be calculated from the resulting concentration/effect curves. We observed that $EC_{50}$ values vary with the concentration of the biofilm suspension used for the tests. This led us to define a normalized $EC_{50}$ which can be used to reliably compare Cd, Cu, Ni, and Zn tolerance levels of biofilms sampled at different sites. Normalized $EC_{50}$ values obtained in a heavily polluted site (Andresy) can be more than 10 times higher than normalized $EC_{50}$ values obtained at Saint-Maurice, which is less polluted although it is still located in an urban area (Tusseau-Vuillemin et al. 2007). Therefore metal tolerance of periphyton can be simply assessed by metal toxicity testing using $\beta$-glucosidase activity as an endpoint and uncomplicated biomass measurements by filtration for TSS normalization.

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