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Diffuse urban pollution increases metal tolerance of natural heterotrophic biofilms

Lise C. Fechner [a,b,*], Catherine Gourlay-Francé [a,b], Adeline Bourgeault [a,b], Marie-Hélène Tusseau-Vuillemin [c]

[a] Cemagref- Unité de Recherche Hydrosystèmes et Bioprocédés, Parc de Tourvoie, BP 44, F 92163 Antony Cedex, France
[b] FIRE, FR-3020, 4 Place Jussieu 75005, Paris, France
[c] IFREMER, Technopolis 40, 155 Rue Jean-Jacques Rousseau, 92138 Issy-Les-Moulineaux, France

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ABSTRACT

This study is a first attempt to investigate the impact of urban contamination on metal tolerance of heterotrophic river biofilms using a short-term test based on β-glucosidase activity. Tolerance levels to Cu, Cd, Zn, Ni and Pb were evaluated for biofilms collected at three sites along an urban gradient in the Seine river (France). Metallic pollution increased along the river, but concentrations remained low compared to environmental quality standards. Biofilm metal tolerance increased downstream from the urban area. Multivariate analysis confirmed the correlation between tolerance and contamination and between multi-metallic and physico-chemical gradients. Therefore, tolerance levels have to be interpreted in relation to the whole chemical and physical characteristics and not solely metal exposure. We conclude that community tolerance is a sensitive biological response to urban pressure and that mixtures of contaminants at levels lower than quality standards might have a significant impact on periphytic communities.

1. Introduction

Over the past decades, human activities in urban areas have contributed to the increase of chemical contamination of aquatic ecosystems (Meybeck, 2003). Typical urban pollution can be described as a mixture of contaminants at low, sublethal concentrations (Tusseau-Vuillemin et al., 2007). It is difficult to evaluate the long-term biological consequences of such diffuse, chronic contamination as it does not engender easily-detectable biological responses in situ (Beasley and Kneale, 2004; Bourgeault et al., 2010). The distinction between chemical-induced changes and changes due to other causes is not always clear as endpoints used to evaluate toxicity may vary due to environmental and/or biological variability (Blanck et al., 1988; Sabater et al., 2007; Soldo and Behra, 2000). Assessment of the impacts of urban contaminants in the field therefore requires the use of efficient tools to link biological impairment to chemical contamination.

The PICT (Pollution-Induced Community Tolerance) approach was proposed as an efficient ecotoxicological tool to relate tolerance acquisition at the community level to exposure to toxicants (Blanck, 2002; Blanck et al., 1988). It relies on the assumption that sensitive components of the exposed community (species, genotypes or phenotypes) will be replaced by more tolerant ones during exposure, thus leading to an increase of community tolerance. Tolerance development can be measured as a shift in the Effect Concentration (usually EC_{50}) that is obtained with a short-term toxicity test based on a physiological endpoint. In particular, the PICT approach was proposed as a tool to demonstrate causative links between toxicants and their adverse biological effects (tolerance levels), due to its ability to discriminate between selection pressures (Blanck et al., 2009), although co-tolerance phenomenons might confound interpretation of the results (Blanck, 2002).

Biofilms (or periphyton) are complex, natural assemblages of micro-organisms that develop on submerged substrata. They typically consist of bacteria, micro-algae, protozoa and fungi embedded in a polymeric matrix. Because of their short-generation time, these micro-organisms respond quickly to disturbances and are thus regarded as early-warning indicators to detect the effects of toxicants (Sabater et al., 2007). Moreover, biofilms provide a community level perspective on biological responses, which is more ecologically-relevant to assess the impacts of contaminants than data obtained from single-species tests (Clements and Rohr,

* Corresponding author.
E-mail address: lise.fechner@cemagref.fr (L.C. Fechner).

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2009; Lehmann et al., 1999; McClellan et al., 2008). The PICT methodology has been applied using natural biofilms to link tolerance levels to river contamination for instance with herbicides (Pesce et al., 2010), using photosynthetic activity as endpoint in toxicity tests, or with Zn, with both photosynthetic and bacterial activity (measured as thymidine incorporation), as endpoints (Blanck et al., 2003). Yet ecotoxicological investigations attempting to link the biological responses of collected organisms to the presence of stressors in the field remain scarce (Clements et al., 2002).

Most PICT studies have focused on tolerance measurements of phototrophic algal communities (periphyton or phytoplankton) exposed to herbicides or Cu (for instance Blanck et al., 2009; Guasch et al., 1998; McClellan et al., 2008; Serra et al., 2010). However, in urban fluvial ecosystems, heterotrophic communities play an important ecological role, in particular in bio-mineralization processes. Hence, exo-enzymes, which control essential metabolic pathways, are interesting to study the effects of environmental perturbation on the functioning of aquatic ecosystems (Admiraal and Tubbing, 1991). In particular, β-glucosidase activity represents the activity of heterotrophic micro-organisms (Chrost, 1991). It is therefore an interesting endpoint for short-term tests designed to measure the tolerance of heterotrophic communities. Moreover, several toxicity tests used in PICT studies are performed using radioactive reagents such as labeled-thymidine for measurements of bacterial growth or labeled-CO_2 for photosynthesis measurements (see for instance Blanck et al., 2003). β-glucosidase activity, in contrast, can be measured using non-toxic fluorescent susbtrates. Recently, it was successfully used as the endpoint in toxicity tests measuring the tolerance to metals of natural freshwater biofilms (Fechner et al., 2010a). Additionally, this short-term test was shown to be a fast and sensitive means to measure biological effects at the community level in microcosms' experiments in which biofilms were exposed to metals (Cd, Ni and Zn) at environmental levels (Fechner et al., 2011a). Since sewers and road and roof run-offs are major metal contamination sources (Thévenot et al., 2007), metals are important to consider when trying to assess the impact of urban contaminations.

The objective of our study is to detect pollution-induced effects on fluvial biofilms collected in a typical urban river (the Seine river, France), under chronic, multi-metallic exposure at low, environmental concentrations. For that purpose, we used the recently-developed short-term toxicity test based on β-glucosidase activity to detect differences in Cu, Cd, Ni, Pb and Zn tolerance levels of the heterotrophic component of fluvial biofilms collected in situ.

2. Material and methods

Biofilms were collected along the Seine river (France) upstream (site 1) and downstream (sites 2 and 3) from the Paris urban area after several weeks of in situ colonization. Biofilms were analyzed using general descriptors such as dry weight (DW), ash-free dry weight (AFDW) and chlorophyll *a* (Chl-a) concentrations and biofilm tolerance to metals (Cu, Cd, Ni, Pb and Zn) was evaluated using short-term toxicity tests based on β-glucosidase activity. Modifications of both bacterial and eukaryotic community structures were also assessed with ARISA (*Automated Ribosomal Intergenic Spacer Analysis*) fingerprints. Multivariate analysis was then used to study the correlations between tolerance and metal contamination.

2.1. Study sites

Biofilms were collected on three sites located along the Seine river (North of France). Site 1 (Marnay-sur-Seine) was located in a non-urbanized area, approximately 200 km upstream from Paris (Strahler: 6, median annual flow 50 m^3/s), and sites 2 (Bougival, Strahler: 7, mean annual flow 184 m^3/s) and 3 (Triel-sur-Seine, Strahler: 8, mean annual flow 340 m^3/s) were both downstream from Paris. In particular, site 3 was located about 20 km downstream from the discharge of the sewer treatment plant Seine-Aval (nominal capacity 8 10^6 inhabitants, flow 24 m^3/s). Total, dissolved and labile metal concentrations, as well as 15 physico-chemical parameters were monitored monthly at the three sites over one year (Priadi et al., 2011). Only the data collected over the period corresponding to this study (3 samplings from May to July 2009, with one sample for total and dissolved metal concentrations and for physico-chemical parameters at each sampling date) was used. P–PO_4 and SiO_2 concentrations were measured once (July 2009) with the Ascorbic Acid Method and the Silicomolybdate Method respectively. Each labile metal concentration corresponds to three Diffusive Gradients in Thin films (DGTs) (Zhang and Davison, 1995) immersed in the river during one month (Priadi et al., 2011). DGTs provide an estimate of time-weighted average concentrations of inorganic and weakly-complexed dissolved metals (Tusseau-Vuillemin et al., 2007).

2.2. Collection of periphytic communities

Biofilms were grown on Low Density PolyEthylene membranes (approximately 15×30 cm^2) vertically attached to plastic crates as in Fechner et al. (2010a). On day 0, 10 membranes were immersed in the river at each site. After 28, 35 and 42 days of colonization, 2 to 3 colonized membranes were collected from each sampling site and carried back to the laboratory in 250 mL glass-bottles placed in a cooler. Membranes were then hand-scraped to collect periphyton and make biofilm suspensions in mineral water (Grand Barbier, Mont-Dore, France) 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 bacterial and eukaryotic Automated Ribosomal Intergenic Spacer Analysis (ARISA) fingerprints of each biofilm sample, aliquots of biofilm suspensions were centrifuged (15 min, 10 000*g*, 4 °C). Supernatants were discarded and pellets were stored at −80 °C for further use.

2.3. Biofilm chemical characterization

At each sampling date, biofilm DWs, AFDWs and Chl-a concentrations were measured in triplicate as in Fechner et al. (2010b). C/N ratios were calculated from the particulate organic carbon and nitrogen (POC and PON) concentrations in the biofilm suspensions. POC and PON were determined from biofilm pellets (centrifugation: 2000*g*, 15 min), after acidic digestion (HCl, 10%), with a carbon analyzer (VarioELIII, Elementar, Germany) in triplicate. Concentrations of total accumulated metals were measured in the biofilms collected on day 35 as in (Fechner et al., 2011a). Accumulated metal concentrations were analyzed with an Inductively-Coupled Plasma-Mass Spectrophotometer (ThermoFisher Scientific). The accuracy and the precision of the measuring device were regularly controlled with certified reference natural water samples (NIST 1640).

2.4. ARISA fingerprinting of biofilms

ARISA fingerprints were obtained at each sampling date as in Fechner et al. (2010b). Briefly, DNA was extracted from biofilm pellets using the Power Soil DNA Isolation Kit (Mobio Laboratories, Inc., Carlsbad, US). PCR amplification of bacterial 16S–23S ITS was performed using primers ITSF/ITSReub (Cardinale et al., 2004) and amplification of eukaryotic ITS1-5.8S-ITS2 regions was performed using primers 2234C/3126T (set euk, appropriate to study eukaryotes: algae, ciliates, etc.) (Ranjard et al., 2001) and ARAlg18S/ITS4 (Fechner et al., 2010b) (set diat, more specific to diatoms). Amplicons were separated on an electrophoresis Bioanalyzer (2100 Electrophoresis Bioanalyzer, Agilent Technologies) and fluorescence data was converted into electrophoregrams using 2100 Expert software (Agilent Technologies). Electrophoregrams were processed using the StatFingerprints R package (Michelland et al., 2009). To compare efficiently fingerprint profiles, the area under each curve was normalized to 1 and proximities between pairs of fingerprinting profiles were calculated using the Manhattan distance (Lear and Lewis, 2009). The distance matrix thus constructed was analyzed using nMDS – non metric multidimensional scaling – in order to visualize similarities in community structure in 2D. NMDS constructs a two-dimensional map showing each fingerprint (ARISA profile) 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 map) and the actual distances. A stress value <0.1 corresponds to a good ordination (Clarke, 1993).

2.5. Tolerance measurements

The tolerance to metals (Cd, Cu, Ni, Pb, Zn) of heterotrophic biofilm micro-organisms was assessed using the normalized EC_{50} values obtained with the toxicity test based on β-glucosidase activity developed by Fechner et al. (2011a; 2010a). Briefly, biofilms were exposed for 1 h to acute concentrations of metals (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). After 1 h of exposure, β-glucosidase activity of the metal-exposed biofilms was measured spectrofluorometrically using Methylumbelliferyl-β-D-glucopyranoside (MUF-GLU) (Sigma–Aldrich). Fluorescence of 4-Methyllumbelliferone (MUF) was measured using an LB 941 Tristar Ti fluorescence microplate reader (Berthold Technologies, Bad Wildbad, Germany) (excitation/emission filters: 355 and 460 nm). Fluorescence measurements were converted into MUF concentrations