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Antioxidant enzyme activities in biofilms as biomarker of Zn pollution in a natural system: An active bio-monitoring study

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ABSTRACT

This study aimed to explore the use of antioxidant enzyme activities (AEA) and biofilm metal accumulation capacity in natural communities as effect-based indicator of metal exposure in fluvial systems. To achieve these objectives, an active biomonitoring using fluvial biofilm communities was performed during 5 weeks. Biofilm was colonized over artificial substrata in a non-polluted site. After 5 weeks, biofilms were translocated to four different sites with different metal pollution in the same stream. The evolution of environmental parameters as well as biofilm responses was analysed over time.

Physicochemical parameters were different between sampling times as well as between the most polluted site and the less polluted ones, mainly due to Zn pollution. In contrast, AEA and metal accumulation in biofilms allowed us to discriminate the high and moderate metal pollution sites from the rest. Zn, the metal with the highest contribution to potential toxicity, presented a fast and high accumulation capacity in biofilms. According to the multivariate analysis, AEA showed different responses. While catalase (CAT) and ascorbate peroxidase (APX) variability was mainly attributed to environmental stress (pH, temperature and phosphate concentration), glutathione-S-transferase (GST) changes were related to metal pollution. Glutathione reductase (GR) and superoxide dismutase (SOD) responses were related to both stress factors.

AEA and metal accumulation are proposed as sensitive effect-based field methods, to evaluate biofilm responses after acute metal exposure (e.g. an accidental spill) due to their capacity to respond after few hours, but also in routinely monitoring due to their persistent changes after few weeks of exposure. These tools could improve the Common Implementation Strategy (CIS) of the Water Framework Directive (WFD) as expert group request.

1. Introduction

In 2009, the Common Implementation Strategy (CIS) of the Water Framework Directive (WFD) established the expert group Chemical Monitoring and Emerging Pollutants (CMEP). This expert group was established to take up the challenges arising from the revision of the list of priority substances as well as from the requirements stemming from Directive 2009/90/EC (also called QA/QC Directive). In particular, CMEP has to deal with aspects related to standardization and quality assurance issues stemming from the implementation of Commission Directive 2009/90/EC, such as methods to assess bioavailability for metals. A specific task of the CMEP activity is to elaborate specific guidance on the use of alternative effect-based monitoring methods, such as the use of biomarkers. These methods are required for investigative monitoring and to better evaluate the link between the chemical and ecological status and the effects of the mixture of pollutants and emerging pollutants. Moreover, they are needed to better understand the real environmental quality of the aquatic ecosystems through the evaluation and detection of the effects of pollutants on the aquatic organism, which can detect pollutants that cannot be detected with routine chemical analysis (EC Guidance document no. 25).

Zn, one of the most widespread metals due to both natural and anthropogenic causes, is present in a wide range of concentrations in aquatic systems. Zn concentrations which exceed levels marked by the European and American legislation are often found. In Spain, Zn occurrence led to the establishment of Zn Environmental Quality Standards (EQS) (annual average values (AA)) of 30 $\mu g\ L^{-1}$ for soft water and 500 $\mu g\ L^{-1}$ for hard water (from RD 60/2011 by means of the Water Framework Directive (WFD)

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(Directive 2000/60/EC)). The maximum concentration for water quality criteria in the USA is within this range; 120 $\mu g\ L^{-1}$ according to the Environmental Protection Agency (EPA, 2006).

Zn, apart from being a micronutrient, can also be toxic at higher concentrations when accumulated in the biota. Its toxic effects have been largely reported in cultures, microcosms and natural systems (Shehata et al., 1999; Behra et al., 2002; Morin et al., 2007; Guasch et al., 2010; Corcoll et al., 2012). Oxidative stress of Zn has also been reported due to its capacity to induce the production of reactive oxygen species (ROS) (Sauser et al., 1997; Nagalaskshmi and Prasad, 1998; Collén et al., 2003; Valavanidis et al., 2006; Tripathi et al., 2006; Qian et al., 2011; Guasch et al., 2010; Bonnineau et al., 2011).

Antioxidant enzyme activities (AEA) are common mechanisms in the organism to regulate the ROS produced by organisms as a result of metabolic processes, such as respiration or photosynthesis (Asada, 2006). Furthermore, AEA play an important role in cellular defence strategy against oxidative stress caused by toxicants like metals (Collén et al., 2003; Tripathi et al., 2006; Valavanidis et al., 2006). Since AEA are able to respond to acute and chronic metal exposure, some authors have proposed their use at both temporal scales (Valavanidis et al., 2006; Guasch et al., 2010; Maharana et al., 2010; Bonnineau et al., 2010). In addition, AEA require less time to be analysed than taxonomy (Bonet et al., 2012).

AEA response to stress is usually not linear and may also change after chronic exposure due to adaptation. Bonnineau (2011) suggested that AEA follow a unimodal pattern with different patterns of activity: increase, saturation, decrease and inhibition. Most AEA studies have been performed in cultures and with single species in the field (Sauser et al., 1997; Geoffroy et al., 2004, Li et al., 2006; Tripathi et al., 2006; Pereira et al., 2009), and with less frequency in microcosm systems with complex communities, like biofilms (Bonnineau et al., 2010; Guasch et al., 2010; Bonnineau et al., 2011; Bonet et al., 2012). However, they offer a poor and lower level of ecological realism (Clements and Newman, 2002).The use of biofilms as bioindicators is due to their capacity to respond to organic and inorganic pollution after both acute and chronic exposure (Soldo and Behra, 2000; Morin et al., 2007; Sabater et al., 2007; Dorigo et al., 2010; Romaní, 2010). Few studies, to our knowledge, have been carried out in natural freshwater systems using biofilms to evaluate AEA responses to metal pollution (Bonnineau et al., 2010; Bonet et al., 2013).

AEA responses are dependent on time, dose, toxicant, as well as on organisms (from single species to complex communities). Hence, their interpretation can be complex. In Bonet et al. (submitted for publication), which was partly conducted in the same stream as this study, we showed an induction of CAT and GST after a short active bio-monitoring experiment (6 and 24 h). In contrast, higher metal contamination in Riou Mort (SW France) reduced all the AEA analysed (CAT, APX, GR and GST). Under chronic conditions, during an annual monitoring in this stream (the Riera d'Osor), AEA, like the algal biofilm community, showed an interesting seasonal variation in sites with no or low levels of metal pollution, whereas this seasonality was reduced in the most polluted site. Furthermore, it showed that glutathione-S-transferase (GST) was the most sensitive AEA to Zn pollution changes over the year (Bonet et al., 2013). Changes in autotrophic and heterotrophic biofilm communities, attributed to Zn pollution, were also reported in Tlili et al. (2011) and Corcoll et al. (2012).

In contrast to AEA patterns over time, biofilm metal accumulation in microcosm experiments was linear (Bonet et al., 2012) over a 5-week period. However, do biofilms have an unlimited metal accumulation capacity under natural conditions with uncontrolled abiotic factors (e.g. temperature, hardness, and pH)? Can biofilms accumulate all metals present in water and do the accumulation kinetics depend on metal form (free, labile or dissolved)?

Hence, in this study we explore, on the one hand, AEA responses to metal pollution and, on the other, biofilm metal accumulation capacity to validate their applicability as tools in an effect-based monitoring method as demanded by CMEP. To achieve both objectives a translocation experiment was performed in a metal-polluted fluvial stream, the Riera d'Osor. Natural communities (fluvial biofilms) were translocated from one site to another, allowing their biological responses to be quantified (De Kock and Kramer, 1994). This approximation provides a high degree of realism in ecotoxicology (Ivorra et al., 1999) and allows stress responses under complex aquatic field situations to be evaluated (Rotter et al., 2011). Biofilms were translocated from a colonization site to different sites with different metal pollutions. Physicochemical and biological parameters of each site were analysed to know the main differences between and within the reference site and polluted sites after translocation. Specifically, the set of AEA analysed was: catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione-S-transferase (GST) and superoxide dismutase (SOD). Due to differences between sites, mainly attributed to metal concentration, we expected to observe different magnitudes and types of biological responses and metal accumulation capacities. We also expected that functional responses would occur in a short period of time after translocation. After some days, when biofilm adapted to metal pollution, stable AEA were expected in polluted sites, but in different ones from the reference site due to differences in metal exposure history. Biofilm metal accumulation capacity would tend to increase over time, and differences between metals were also expected.

2. Material and methods

2.1. Study site

Translocations were conducted in the Riera d'Osor. A 5 km stretch was selected starting after the village of Osor and ending before the town of Anglès, including the mining area (Fig. 1A). The Riera d'Osor is a small tributary of the river Ter located in the north east of Catalonia (NE Spain) (Fig. 1A). This stream is affected by effluents and runoffs from a former mine that extracted sphalerite ((Zn, Fe)S) and galena (PbS). In spite of the fact that mining activities finished in 1980, no environmental rehabilitation was carried out and the stream is still receiving the input of a continuous mine effluent (referred to in the text and Fig. 1A as mine source (MS)) and also diffuse metal inputs from mine run-over of metal-polluted landfills. Despite metal pollution, this stream is relatively well preserved, with well-developed riparian vegetation and low urban pressures. Its hydrology has been altered due to the diversion of part of the stream water flow for electric power production.

Four sites with different metal concentrations were selected: the non-metal polluted site called *upstream*, before the mine source, as the reference site (referred to as Up), *mining 1* (referred to as M1) placed after Up site and upstream of the main source, *mining 2* (referred to as M2), located just after the mine source with continuous inputs of metals, and *mining 3* (referred to as M3) further downstream with less metal concentrations due to metal precipitation and storage in the sediment (Tlili et al., 2011; Corcoll et al., 2012).

2.2. Experimental set-up and sample collection

The experiment started on 12 April and lasted until 21 June, 2009. Biofilm was grown on artificial substrates to reduce the heterogeneity that occurs on natural substrates (Cattaneo et al., 1997). Small (1.2 × 1.2 cm) and large (8.5 × 2.0 cm) sand-blasted glass substrata were fixed to cement cobbles (40 × 40 cm) with silicon sealant. These cement cobbles were placed horizontally on the streambed in the colonization site (CS), in the middle of the stream (Fig. 1A). After 5 weeks of biofilm colonization, artificial substrata were moved from CS to the four selected sites (Up, M1, M2 and M3) in similar light and water current conditions. After translocations, water and biofilm samples were collected after 6 h, 1, 3, 7, 21 and 35 days. To avoid translocation effects being confused, Up site, with similar physicochemical conditions and with a good ecological status like CS, was used as the reference site to follow the biological evolution of translocated biofilms (Fig. 1B). With this translocation design, different changes in Zn exposure were achieved: (i) low Zn pollution in M1, (ii) moderate Zn pollution in M3 and (iii) high Zn pollution in M2. No rain was expected during the translocation experiment. However, a heavy rain event of 46 mm occurred (according to Met Office, 2007) just 3 days before.