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The use of antioxidant enzymes in freshwater biofilms: Temporal variability vs. toxicological responses

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ABSTRACT

This study aims to investigate the potential of antioxidant enzyme activities (AEA) as biomarkers of oxidative stress in freshwater biofilms. Therefore, biofilms were grown in channels for 38 days and then exposed to different concentrations (0–150 $\mu g\,L^{-1}$) of the herbicide oxyfluorfen for 5 more weeks. Under control conditions, the AEA of biofilms were found to change throughout time with a significant increase in ascorbate peroxidase (APX) activity during the exponential growth and a more important role of catalase (CAT) and glutathione reductase (GR) activities during the slow growth phase. Chronic exposure to oxyfluorfen led to slight variations in AEA, however, the ranges of variability of AEA in controls and exposed communities were similar, highlighting the difficulty of a direct interpretation of AEA values. After 5 weeks of exposure to oxyfluorfen, no clear effects were observed on chl-*a* concentration or on the composition of other pigments suggesting that algal group composition was not affected. Eukaryotic communities were structured clearly by toxicant concentration and both eukaryotic and bacterial richness were reduced in communities exposed to the highest concentration. In addition, during acute exposure tests performed at the end of the chronic exposure, biofilms chronically exposed to 75 and 150 $\mu g\,L^{-1}$ oxyfluorfen showed a higher CAT activity than controls. Chronic exposure to oxyfluorfen provoked then structural changes but also functional changes in the capacity of biofilm CAT activity to respond to a sudden increase in concentration, suggesting a selection of species with higher antioxidant capacity. This study highlighted the difficulty of interpretation of AEA values due to their temporal variation and to the absence of absolute threshold value indicative of oxidative stress induced by contaminants. Nevertheless, the determination of AEA pattern throughout acute exposure test is of high interest to compare oxidative stress levels undergone by different biofilm communities and thus determine their antioxidant capacity.

1. Introduction

In freshwater ecosystems, biofilm communities are now recognized as pertinent indicators of perturbations (Sabater and Admiraal, 2005). These complex communities, composed of algae, bacteria, fungi, and protozoa, are embedded in a matrix constituted by extra-polymeric substances (EPS). They live attached to different types of substrates (cobbles, wood, sand, etc.) and are the main primary producers in open streams (Romaní, 2010; Stevenson et al., 1996). To assess biofilm status, different structural and functional variables are usually determined. They include community composition (mostly of diatoms), photosynthesis, biomass and heterotrophic activity (Sabater et al., 2007; Weitzel, 1979). To complete the information given by these indicators, we propose the use of antioxidant enzyme activities (AEA) in biofilms as indicators of oxidative stress induced by toxicants. In fact antioxidant enzymes participate in the regulation of reactive oxygen species (ROS) levels to avoid their accumulation and the resulting oxidative stress (Mittler, 2002). Previous studies highlighted the interest of AEA as sensitive markers of stress induced by organic and inorganic toxicants. Dewez et al. showed that the catalase (CAT) activity was a more sensitive biomarker of fludioxonil toxicity than photosynthetic parameters in *Scenedesmus obliquus* (Dewez et al., 2005). In freshwater biofilms, AEA were found to be more sensitive to

Abbreviations: EPS, extra-polymeric substances; AEA, antioxidant enzyme activities; ROS, reactive oxygen species; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase.

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copper toxicity than photosynthetic parameters (Guasch et al., 2010). The present study focused on three important antioxidant enzymes: CAT, ascorbate peroxidase (APX) and glutathione reductase (GR). CAT (EC 1.11.1.6) and APX (EC 1.11.1.11) catalyse the transformation of hydrogen peroxide in water and oxygen mainly in peroxisomes and chloroplast, respectively (Chelikani et al., 2004; Lesser, 2006). GR (1.8.1.7) participates in this reaction by regenerating the cofactor needed by APX (ascorbate-glutathione cycle, Mittler, 2002).

Since temporal variations affect function and structure of communities strongly, functional biomarkers chosen to reflect perturbations, such as AEA, may also change due to temporal variability. Indeed, biofilms are very dynamic communities in which changes in biomass due to processes of attachment, colonization, exponential growth, senescence and sloughing (Biggs, 1996) are concomitant to species succession (Hudon and Bourget, 1981; Peterson and Stevenson, 1990). These processes are linked to changes in community functioning. For instance, Sabater and Romaní (1996) found a higher respiratory activity in younger rather than in mature biofilms from an undisturbed Mediterranean stream. Romaní et al. (2008) also observed that the release of extracellular bacterial enzymes allowing organic matter compound degradation in the EPS matrix was higher at the beginning of the biofilm formation than at the end of colonization. Though, in ecotoxicology, the effect of these temporal variations is reduced by using same age communities (Clements and Newman, 2002), the temporal variation represents an estimate of the "natural" range of variation and thus may still be a pertinent scale to interpret the importance of further variations related to disturbances. Since enzymes are sensitive to different factors (e.g. pH, temperature), changes in biofilm environmental conditions due to growth are expected to provoke variations in AEA. To our knowledge, patterns of AEA in freshwater biofilms throughout time are unknown. Thus, the first aim of this study was to determine the "temporal" range of variation of AEA of the biofilm. This background information is essential to interpret the importance of AEA's variations in response to chemical exposure and thus to use AEAs as biomarkers of oxidative stress.

In the present study, temporal changes in AEA of non-exposed biofilms were investigated on mature biofilm communities established after several weeks of colonization, during their transition from an exponential to a slow growth phase. Mature communities were used because ecotoxicological tests are better performed on those communities to test toxicant impact on a fully active community (Clements and Newman, 2002). The study of the temporal variability of AEA in non-exposed communities was complemented with more traditional biofilm metrics, such as biomass variables, photosynthetic parameters (Sabater et al., 2007), relative abundance of the different algal groups (based on marker pigments; Jeffrey et al., 1997) and some antioxidant pigments (e.g. carotenoids; Pinto et al., 2003).

In the second part of this study, the temporal variability of AEA observed in a control situation was compared to the variations of AEA in response to contamination during acute and chronic ecotoxicological tests. For these tests, the herbicide oxyfluorfen was selected since it is representative of compounds likely to be tested in ecotoxicological tests and it is expected to provoke oxidative stress. Indeed, this diphenyl-ether herbicide inhibits chlorophyll-*a* biosynthesis and provokes the accumulation in the cytoplasm of protoporphyrin IX, a potent photo-sensitizer that generates high levels of singlet oxygen and so oxidative stress (Aizawa and Brown, 1999; Duke et al., 1991). Though its use has been recently re-approved by the UE, oxyfluorfen exposure has been shown to provoke oxidative stress in algae (Geoffroy et al., 2003; Kunert et al., 1985; Sandmann and Böger, 1983) and cyanobacteria (Sheeba et al., 2011). Consequently, the European Food Safety Authority (EFSA) pointed out the high risk for algae by this compound as well as the need for further studies on its potential impact on aquatic organisms (EFSA, 2010). Based on these previous studies, oxyfluorfen is expected to provoke oxidative stress and so changes in AEA also in biofilms, but has never been tested. In the present study, changes in AEA after acute and chronic exposure to oxyfluorfen were compared to changes in more traditional metrics as described earlier. In addition, after five weeks of exposure, the structure (bacterial/eukaryotic diversity, algal composition) and the function (AEA response in short-term toxicity tests) of the exposed and control communities were compared to assess whether AEA plays a role in the selection of more resistant species expected to occur under chronic exposure of a community to a critical level of contaminant.

The objectives of the present study were then:

1. to characterize the pattern of temporal variation of AEA in mature biofilms.
2. to compare the temporal variation of AEA and the toxicological variation provoked by the exposure to oxyfluorfen, a toxicant inducing oxidative stress in mature biofilms.
3. to determine the influence of chronic exposure on the capacity of biofilms to respond to a sudden increase in oxidative stress (induced by oxyfluorfen).

2. Materials and methods

2.1. Microcosm setup

Colonization and exposure were performed in an indoor microcosm system consisting of 7 recirculating channels previously described by Serra et al. (2009a). Biofilms were allowed to colonize sandblasted glass substrata of 1.4 and 17 cm^2 installed in the bottom of each channel. In each channel, 10 L of dechlorinated tap water was used as a culture medium and changed 3 times a week; aquarium pumps allowed water recirculation. At each water renewal, phosphate was added to a final nominal concentration of 30 $\mu g\,L^{-1}$ to avoid nutrient depletion and P limitation. A cooling bath maintained the water temperature at 20 °C. Once a week during the first 5 weeks of colonization, an original inoculum of biofilm collected, during the Spring season, from the river Llémana (NE Spain, Serra et al., 2009a) was added to each channel. Light was provided by halogen lamps (80–120 μmol photons $m^{-2}\,s^{-1}$) with a light regime of 12 h:12 h light:dark.

After 5 weeks of colonization, on day 38, biofilms were exposed to increasing concentrations of oxyfluorfen (CAS: 42874-03-3) following an exponential design (Ricart et al., 2009). Three channels were used as controls and the remaining 5 channels were exposed to 3, 7.5, 15, 75 or 150 $\mu g\,L^{-1}$ of oxyfluorfen. Oxyfluorfen was added in each channel from a stock solution at 15 $mg\,L^{-1}$ in 2.5% acetone to obtain 0.025% acetone in each channel, acetone was also added in a similar amount in control channels. At each water renewal, toxicant and/or acetone (when appropriate) were added to compensate for potential degradation of the toxicant and to ensure a maximal exposure.

To characterize the temporal pattern of AEA and to link it with changes in other biological variables, the 3 control channels were sampled on days 33, 36, 38, 39, 41, 59, 66 and 73. At each sampling and from each control channel, 3 samples (each consisting of three 1.4 cm^2 glass substrata) were collected randomly for AEA measurements, 5 samples (1.4 cm^2 glass substrata each) for the measurement of photosynthetic efficiency and 3 samples (1.4 cm^2 glass substrata each) for pigment analyses.

To determine the variability of AEA due to contaminant exposure, AEA but also photosynthetic efficiency, protein content and wet weight of biofilms from all channels were measured after