Catalase in fluvial biofilms:

a comparison between different extraction methods and example of application in a metal-polluted river

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Abstract

Antioxidant enzymes are involved in important processes of cell detoxification during oxidative stress and have, therefore, been used as biomarkers in algae. Nevertheless, their limited use in fluvial biofilms may be due to the complexity of such communities. Here, a comparison between different extraction methods was performed to obtain a reliable method for catalase extraction from fluvial biofilms. Homogenization followed by glass bead disruption appeared to be the best compromise for catalase extraction. This method was then applied to a field study in a metal-polluted stream (Riou Mort, France). The most polluted sites were characterized by a catalase activity 4 to 6 times lower than in the low-polluted site. Results of the comparison process and its application are promising for the use of catalase activity as an early warning biomarker of toxicity using biofilms in the laboratory and in the field.

Keywords: biofilm, catalase, extraction, biomarker, metal.

1. Introduction

Oxidative stress is a common form of stress due to the accumulation of reactive oxygen species (ROS) in cells. H_2O_2 is a ROS produced by organisms in normal metabolism such as photosynthesis or respiration but also in stress metabolism induced by natural or chemical disturbances (Mittler 2002). In cells, levels of H_2O_2 have to be tightly regulated. On the one hand, H_2O_2 accumulation may cause great damage to the cell due to its high capacity of unrestricted oxidation (Edreva 2005). On the other hand, H_2O_2 is an essential signalling molecule in different pathways as, for instance, in defence reactions against pathogens or regulation of cell expansion in higher plants (Laloi et al. 2004). Among the antioxidant mechanisms developed by cells to maintain redox homeostasis, the antioxidant enzyme catalase (CAT, EC 1.11.1.6) is one of the most efficient enzymes in degrading hydrogen peroxide (H_2O_2) in water and oxygen (Lesser 2006) and has been extensively studied (Chelikani et al. 2004). CAT enzymes are predominantly located in peroxisomes of the cells (De Duve and Baudhuin 1966; Stabenau 1984). Due to its relatively low affinity for H_2O_2, CAT is mainly involved in the removal of excess H_2O_2 during important oxidative stress (Mittler 2002). As a change in redox balance can be dramatic for the cell, changes in CAT level may reflect environmental disturbances. CAT inhibition may suggest the presence of disturbances that reduce the cell's ability to cope with oxidative stress, while an increase in CAT activity may suggest an active response of cells to oxidative stress. Both CAT inhibition and activation provide useful information to detect environmental disturbances and estimate cell resistance to oxidative stress. CAT activity may be inhibited by different factors, such as a very high concentration of H_2O_2 (Lardinois et al. 1996) or an excess of light. Moreover, environmental perturbations (osmotic stress, changes in temperature) can reduce the usually high turnover of CAT and, therefore, decrease its activity (Lesser 2006). CAT activity, like other antioxidant enzyme activities, may complement the information obtained by classical endpoints (growth, mortality) and be successfully used in toxicity assessment

in algae (Contreras et al. 2009; Geoffroy et al. 2004; Liu and Xiong 2009; Nie et al. 2009; Qian et al. 2008, 2009). For example, Dewez et al. (2005) pointed out that CAT was a more sensitive biomarker of fludioxionil toxicity than photosynthetic parameters in *Scenedesmus obliquus* (Dewez et al. 2005). In addition, Geoffroy et al. (2004) highlighted the higher sensitivity of CAT than other antioxidant enzymes (ascorbate peroxidase, glutathione reductase) in *Scenedesmus obliquus* exposed to the herbicide flumioxazin.

Although these studies emphasized the interest of CAT as a biomarker of oxidative stress in toxicity assessment, they were performed on mono-specific cultures of algae and, therefore, do not allow the effects of these toxicants to be understood at community level. Freshwater ecology studies performed at community level (e.g. using freshwater biofilms) provide a more realistic approach to assess the effects of toxicants (Sabater et al. 2007). Biofilms are complex communities composed of green algae, diatoms (brown algae), cyanobacteria, bacteria, protozoa and fungi; these micro--organisms live closely together embedded in an extracellular matrix (Romaní 2010). The extraction of CAT in such a community is challenging due to the diversity in cells walls and micro-organism size. For instance, silicate skeleton and cellulosic cell walls of diatoms and green algae, respectively (Mackie and Preston 1974; Soininen 2007), may be more difficult to break than the cell walls of bacteria composed of peptidoglycans and/or phospholipids (Schleifer and Kandler 1972). An efficient extraction method should maximize the quantitative extraction of CAT from all the different micro-organisms of biofilm communities. To our knowledge, only two previous studies related the use of antioxidant enzymes in biofilms (Bonnineau et al. 2010; Guasch et al. 2010). Although both studies highlighted the potential of antioxidant enzymes as biomarkers of toxicity for copper (Guasch et al. 2010) or for the β-blocker propranolol (Bonnineau et al. 2010), the method used for extraction in these studies (homogenization with a glass tissue grinder) presented limitations. In Guasch et al. (2010) a high amount of biofilm surface (100 cm^2) was required for extraction. The reduction of the amount of biofilm needed for CAT extraction would

improve the feasibility of the method both in the laboratory, where space is limited, and in the field, where biofilm biomass is subject to strong variations (Romaní and Sabater 2001). In the second study, the use of the same extraction method but with less biomass led to highly variable CAT measurements, probably due to a low concentration of CAT in the final extract (Bonnineau et al. 2010). Both examples also illustrate the need to improve the method of CAT extraction in biofilm communities by minimizing the amount of biomass needed and maximizing CAT concentration in enzymatic extracts.

The main objective of the present study was to find an appropriate method for the extraction of CAT from fluvial biofilms. More specifically, the study aimed to:

1. Improve the extraction procedure in order

    - to maximize the quantity of protein extracted and the specific CAT activity in the enzymatic extract

    - to minimize the amount of biomass required.

2. Test the applicability of the selected procedure to assess exposure effects in naturally occurring biofilms.

The present study focused on CAT activity as it is the main antioxidant enzyme in plant (Geoffroy et al. 2003) and has been shown to be especially sensitive to toxicant exposure in algae (Geoffroy et al. 2004). However previous studies had shown the importance of other antioxidant enzymes as biomarkers of oxidative stress (Contreras et al. 2009; Geoffroy et al. 2004; Liu and Xiong 2009; Nie et al. 2009; Qian et al. 2008, 2009). The extraction method developed for CAT is expected to provide basis for the study of other antioxidant enzymes within biofilms.

Different methods (trituration, ultrasonication, homogenization and homogenization followed by glass bead disruption) were selected based on current methods used for antioxidant enzyme extraction in microalgae or bacteria (Choo et al. 2004; Janknegt et al. 2007; Tang et al. 1998; Wang et al. 2006) or used for DNA extraction from soil bacterial communities (Bäckman et al. 2003). These extraction

techniques require a priori a lower amount of biomass and a smaller volume of extraction buffer than the homogenization with a glass tissue grinder used in previous studies on biofilms (Bonnineau et al. 2010; Guasch et al. 2010). Extraction by ultrasonication and by trituration were compared and the best extraction methods between these two was compared to extraction by homogenization and homogenization followed by glass beads disruption. Efficiencies of methods in terms of protein extraction and CAT extraction were compared as well as their reproducibility. The comparison was done using biofilms grown in the laboratory under controlled conditions. CAT activities were then measured in natural biofilms growing in a metal--polluted river (Riou Mort, France) and compared to a more classical endpoint: photosynthetic efficiency.

2. Material and methods

2.1 Method comparison in laboratory biofilms

The comparison between the different methods of extraction was performed with laboratory biofilms under realistic experimental conditions. After colonization, biofilms were briefly exposed either to their colonization light intensity or to strong light intensity and after exposure they were sampled for CAT extraction. As CAT is subject to photoinhibition, strong light intensity exposure is expected to reduce its activity (Feierabend and Engel 1986). The sensitivity of the extraction methods: trituration, homogenization and homogenization followed by glass bead disruption, for detecting CAT activity under such an extreme situation was also tested.

2.1.1 Biofilm colonization

Colonization was performed in crystallizing dishes as described previously (Bonnineau et al. 2010). Briefly, biofilm communities colonized 1 cm^2 of sandblasted glass substrata installed in crystallizing dishes of 2 L. During the entire colonization process, an aquarium pump enabled circulation of water to simulate flowing water at constant velocity. Biofilms were incubated at 19ºC and under a 12/12 h day–night

cycle (120 µmol photons m^{-2} s^{-1}). An inoculum of biofilm, obtained by scraping cobbles from the Llémena River (NE Spain, Serra et al. 2009a), was added weekly to each dish. Dechlorinated tap water was used as culture media and changed twice a week. Nitrogen content of water was at 25 µmol of N L^{-1} (Serra et al. 2009b). Therefore, at each water renewal phosphate was added to a final nominal concentration of 158 µg L^{-1} (1.64 µmol of P L^{-1}) to avoid nutrient depletion and P or N limitation (Hillebrand and Sommer 1999).

2.1.2 Experimental set up

After 3 weeks of colonization, each glass substrata was transferred into a vial containing 10 mL of media. Samples were then incubated for 8h under colonization light intensity or strong light intensity (900 µmol photons $m^{-2}s^{-1}$). The other parameters were similar to colonization. A single-speed orbital mixer (KS260 Basic, IKA®) was used to maintain constant agitation. Control biofilms (referred to as controls) and biofilms exposed to strong light intensity (referred to as exposed biofilms) were sampled after 2 and 8 hours of exposure, one sample consisted of 2 glass substrata of 1 cm^2. Samples exposed to the same treatment and extracted by the same method were considered as experimental replicates. Control biofilms were used to compare extraction methods by trituration (4 replicates) and ultrasonication (3 replicates) while exposed biofilms were used to compare extraction methods by trituration (3 replicates), homogenization (3 replicates) and homogenization and glass beads (2 replicates).

2.1.3 Biofilm sampling

For each sample, the biofilm was removed from the glass substrata with a cell scraper (Nunc, Wiesbaden, Germany) and put into an eppendorf tube. After centrifugation at 2300 g and at 10ºC for 5 min, the excess water was removed, and samples were weighted (wet weight) and frozen immediately in liquid nitrogen. Finally, samples were stored at -80ºC until protein extraction and enzymatic assays had been carried out.

2.1.4 Protein extraction

The present study focused on comparing mechanical extraction techniques and not chemical ones. The same extraction buffer (containing 100 mM Na_2HPO_4/KH_2PO_4, pH 7.4, 100 mM KCl, 1 mM EDTA) was thus used in the different protocols adapted from existing methods. Samples were kept on ice during extraction by the different methods described as follows.

Trituration under liquid nitrogen (adapted from Choo et al. 2004 and Wang et al. 2006): the frozen sample was placed in a mortar and ground to powder with a pestle adding liquid N_2 when needed. Then, the powder was transferred to an eppendorf tube where 200 µL of extraction buffer were added for 100 mg of wet weight of sample.

Ultrasonication (adapted from Janknegt et al. 2007): the frozen sample was resuspended in 400 µL of extraction buffer for 100 mg of wet weight of samples and sonicated applying 2 pulses of 30 s and 25 µm of amplitude (Labsonic 2000, B.BRAUN).

Homogenization (adapted from Bäckman et al. 2003): 200 µL of extraction buffer were added for each 100 mg of wet weight of sample, and then samples were homogenized by applying 2 pulses of 30 s of homogenizer (DIAX900, Heidolph) with 1 min interval on ice.

Homogenization and disruption with glassbeads (adapted from Bäckman et al. 2003 and Tang et al. 1998): after homogenization, as described above, 100 mg of glass beads (≈500µm of diameter) were added for each 100 mg of wet weight of samples and further cell disruption was performed through 3 pulses of 30 s of beadbeater (MP FastPrep-24, v = 4 m s^{-1}) with 5 min intervals on ice.

After cell disruption, homogenates were centrifuged at 10.000 g and 4ºC for 30 min. Supernatants were used as the enzyme source. For each sample, the protein content of supernatant was measured spectrophotometrically (Elx800, BioTek Instruments) in triplicates (referred to as analytical replicates) by the method of

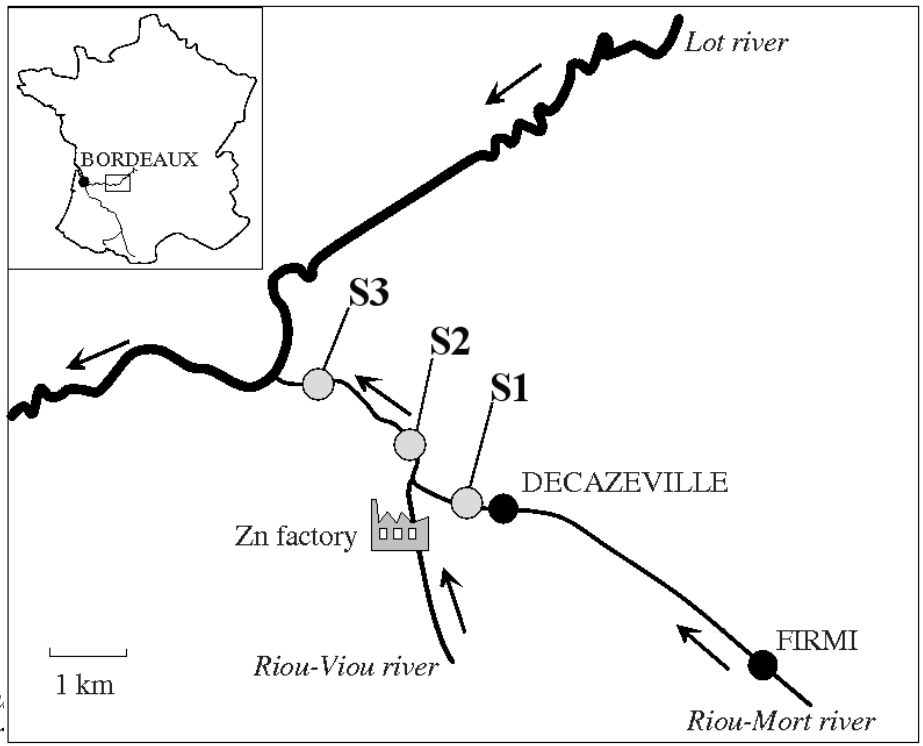
Bradford (Bradford 1976) using dye reagent concentrate from Bio-Rad (Laboratories GmbH, Munich, Germany) and bovine serum albumin as a standard.

2.1.5 Catalase activity measurement

CAT activity was measured spectrophotometrically (UV/Vis Lambda Bio 20, Perkin Elmer) according to Aebi (Aebi 1984) by following the linear decrease in absorbance at 240 nm corresponding to the decomposition of H_2O_2 by CAT. In direct enzymatic assay, substrate concentration has to be in excess throughout the entire assay to avoid limiting the reaction rate. The compromise between substrate concentration and protein quantity defines a linear range of protein quantities for which specific activity is maintained (Palmer 1991). As CAT is inhibited by a high amount of H_2O_2 (Chelikani et al. 2004), the optimal substrate concentration would be the one leading to the highest specific activity and not necessarily the highest one. In this context, a preliminary test with three samples of control biofilm extracted by trituration allowed the optimal substrate concentration and the optimal protein content to be determined. Among the different final concentrations of H_2O_2 tested (2, 10, 15, 20, 25 and 30 mM), 20 mM of H_2O_2 was found to be the optimal one. The three quantities of protein tested (5, 10 and 20 µg) led to similar specific CAT activity and were all in the linear range of protein quantity. Therefore, for each sample, CAT activity was measured in triplicates (referred to as analytical replicates), as follows: the 800 µL reaction mixture contained in a final concentration: 80 mM potassium phosphate buffer (pH 7.0) and the enzyme extract (between 5 and 10 µg of proteins). The reaction was started by adding H_2O_2 at a final concentration of 20 mM. Enzymatic activity was measured after monitoring the decrease in absorbance at 25ºC for 2 min, at the end of which linearity was shown. The specific CAT activity is the amount of H_2O_2 converted per unit of time and per unit of protein in one sample and is expressed in µmol H_2O_2 mg protein^{-1} min $^{-1}$. The total CAT activity is the total amount of H_2O_2 converted per unit of time by one sample and is expressed as µmol H_2O_2 min^{-1} (extinction coefficient: 0.039 cm² µmol^{-1}). Both were calculated in the present study.

2.2 Field study

2.2.1 Study site



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This field study was carried out in the Riou Mort (SW Aveyron department, France), a small tributary of the Lot River located in an industrial basin. This stream is highly contaminated by different metals from its confluence with the Riou-Viou, a stream carrying seepage from a former active zinc factory (Morin et al. 2008). Sampling was performed at three sites, one before the confluence with Riou-Viou (S1, Fig. 1) and two after it (S2, S3, Fig. 1).

2.2.2 Sampling

At each sampling site, biofilm and water samples for metal analysis were collected in triplicate. Water samples (5 mL) were immediately filtered (Whatman nylon filters 0.2 µm) and acidified with 1% of HNO_3 (65 % suprapure, Merck) before

measurements of total dissolved metal concentration. For CAT activity, three samples of biofilm were collected by scraping 4 cm^2 from different cobbles with a cell-scraper (Nunc, Wiesbaden, Germany). Since centrifugation in the field was not possible, the excess of water was eliminated using absorbent paper. Dried samples were frozen immediately in liquid nitrogen, and samples were stored at -80ºC until extraction.

2.2.3 Protein extraction and CAT activity measurement

Protein extraction and quantification were performed as indicated above (2.1.4), the extraction method used was homogenization followed by glass bead disruption. CAT activity measurements were performed in microtiter plates (UV-Star 96 well plate, Greiner®). The use of microtiter plates in enzymatic assay reduces the assay time and allows a high number of samples to be measured in parallel. Moreover, CAT assay in microtiter plates based on the measure of the decrease in absorbance of H_2O_2 has been previously validated with *E. coli* by Li and Schellhorn (2007). Preliminary tests with field biofilms from S1 allowed the optimal substrate concentration and protein quantity to be determined. Among the different final concentrations of H_2O_2 tested (10, 15, 20, 30 and 40 mM), 30 mM of H_2O_2 was found to be the optimal one (leading to the highest specific CAT activity). Among the different quantities of protein tested (0.5, 1, 2, 4, 8 and 10 µg), 2 µg was found to be in the linear range of response of CAT activity. After adaptation to the microtiter plate volume, the 250 µL reaction mixture contained 80 mM of potassium phosphate buffer (pH 7.0) and 2 µg of proteins in a final concentration. Reaction was started by adding H_2O_2 in a final concentration of 30 mM. The decomposition of H_2O_2 was determined by measuring the linear decrease in absorbance at 25ºC for 2 min using a microtiter plate reader Synergy4 (BioTek®). Total (µmol H_2O_2 min^{-1}) and specific (µmol H_2O_2 mg $protein^{-1}$ min^{-1}) CAT activity were calculated.

2.2.4 Measure of photosynthetic efficiency

Estimation of photosynthetic efficiency was done by measuring the chlorophyll–*a* fluorescence of biofilm with a PhytoPAM (Pulse Amplitud Modulated) fluorometer (version EDF, Heinz Walz GmbH). In order to obtain replicate measurements, chlorophyll-*a* fluorescence was measured from three cobbles per site. Measurements were done in vivo in dark conditions; the distance between the optical fiberoptics and the sample surface being set at 4 mm. The fluorescence signal recorded at 665 nm was used to calculate the effective PSII quantum yield parameter according to Genty et al. (1989). The effective PSII quantum yield is an indicator of the efficiency of PSII and is expressed in relative units of fluorescence (r.u.).

2.2.3. Metal analysis

The concentration of dissolved metals in water was determined by inductively coupled plasma mass spectroscopy ICP-MS (7500c Agilent Technologies, Inc. Wilmington, DE). The detection limits were 148.91 µg Al L^{-1}, 70.75 µg Fe L^{-1}, 86.46 µg Zn L^{-1}, 0.00 µg Cd L^{-1}, 0.00 µg Ni L^{-1}, 0.00 µg Cu L^{-1} and 0.00 µg Pb L^{-1}. The accuracy of the analytical method was checked periodically using certified references for water (SPS-SW2 Batch 113, Oslo, Norway), the uncertainties (half width of the 95% confidence intervals) were of 1, 1, 1, 0.02, 0.3, 1 and 0.1 µg L^{-1} for Al, Fe, Zn, Cd, Ni, Cu and Pb, respectively.

2.3 Data analyses

All statistical analyses were done using R 2.6.2 (R development Core Team, 2008). Mean values are always presented with the corresponding standard errors.

2.3.1 Methods comparison in laboratory biofilms

To estimate the influence of sampling time, specific CAT activity of samples collected after 2h and 8h of exposure and extracted by trituration were compared. Statistical differences were tested by a Mann-Whitney test (Bauer 1972; Hollander and Wolfe 1973). To determine the significance of the results, the U value obtained from the test was compared to the critical value of the U-distribution table for $\alpha = 0.1$.

The relationship between the total quantity of protein extracted and the initial wet weight of samples was estimated by calculating the Spearman coefficient and corresponding p-value (Hmisc package, Harrell, 2007).

To compare method efficiencies, differences in total quantity of protein extracted, quantity of protein extracted per wet weight, specific and total CAT activities were tested using the Mann-Whitney test as described earlier. Control samples extracted by ultrasonication and by trituration were first compared, and then exposed samples extracted by trituration, homogenization and homogenization with glass beads, were compared two at a time.

In the present study, the intra-sample variability of the quantity of protein extracted per wet weight and of the specific CAT activity was defined as the closeness of agreement between analytical replicates performed under the same conditions and was measured as the standard deviation divided by the mean of the analytical replicates of one sample and expressed in percentage. The inter-samples variability of each method in terms of quantity of protein extracted per wet weight and specific CAT activity was defined as the closeness of agreement on these variables between samples from a same treatment. Inter-samples variability was measured as the standard deviation divided by the mean of the samples of one method and expressed in percentage.

To estimate light effect on CAT activity, specific CAT activity of control and exposed samples extracted by trituration were also compared by a Mann-Whitney test as explained previously.

2.3.2 Application to field study

To integrate mixture effects of metals present in the field, the cumulative criterion unit (CCU) was calculated for each sampling point as described by Guasch et al. (2009). The following equation was used:

$$CCU = \sum \frac{m_i}{c_i}$$

where m is the dissolved metal concentration and c the criterion value for each metal *i*. According to water hardness in the Riou Mort (around 100 mg $CaCO_3$ L^{-1}), criterion values for each metal were: 1000 µg L^{-1} for Fe, 96 µg L^{-1} for Ni, 106 µg L^{-1} for Zn and 1.1 µg L^{-1} for Cd. Concentrations below detection limits (Al, Cu and Pb) were not included in the calculation. Differences between sampling sites in terms of photosynthetic efficiency, specific CAT activity and CCU were tested by a Mann--Whitney test as explained previously. Spearman coefficient and its associated p-value were calculated to estimate linear correlation between specific CAT activity or photosynthetic efficiency and CCU or metal concentration in water.

3. Results

3.1 Methods comparison in laboratory biofilms

3.1.1 Biofilm colonization

Physical and chemical parameters were stable during colonization. Temperature was 19.7 ± 0.2 ºC, dissolved oxygen concentration: 9.2 ± 0.2 mg L^{-1}, water conductivity: 494 ± 8 µS and pH: 8.4 ± 0.1 (n=36 for all parameters). Water used during this experiment had been previously characterized (Serra et al. 2009b); therefore, only phosphate concentration was measured. Total phosphate depletion was never observed during colonization although phosphate concentrations after water changes (87 ± 12 µg L^{-1}, n=9) declined to low levels before water changes (7 ± 1 µg L^{-1}, n=9).

3.1.2 Sensitivity and repeatability of measurements of protein content and specific CAT activity

The quantity of biofilm obtained after scraping 2 cm^2 of substrata was on average 234 ± 24 mg (n=15) of wet weight. This quantity was sufficient for protein extraction and CAT measurement for all extraction methods tested. A positive correlation was found between the quantity of protein extracted and the wet weight of the samples ($\rho = 0.79$, $p < 0.05$), including results from all methods tested, except those from extraction by ultrasonication (Fig. 2).

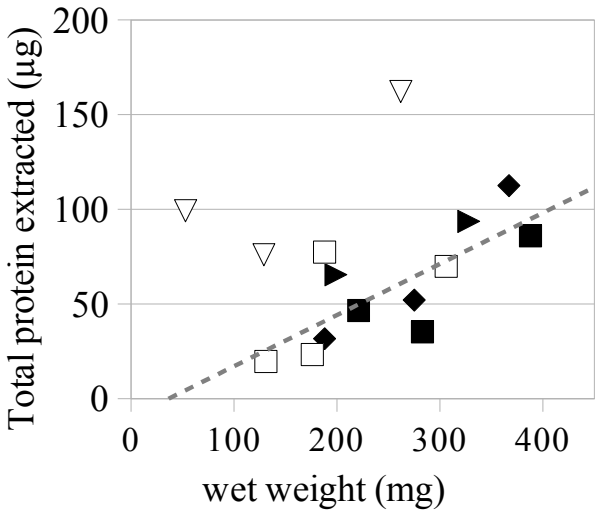

Figure 2. Total protein extracted in function of wet weight of control samples (in white) extracted by trituration (□) or by ultrasonication (∇) and of exposed samples (in black) extracted by trituration (■), homogenization (♦) or homogenization and glass beads (►). The positive correlation between total protein extracted and wet weight of samples extracted by trituration, homogenization and homogenization and glass beads is shown by a grey dotted line.

No significant differences between methods were found for intra-sample variabilities of quantity of protein extracted per wet weight and specific CAT activity ($U > 0$ in all comparisons, $\alpha = 0.1$). For the measure of protein content, intra-sample variability was below 12 % for all methods. For the measure of specific CAT activity, intra-sample variability was below or around 10 % for all methods except for samples extracted by ultrasonication (intra-sample variability = 20.2 ± 11.9 %). This relatively high percentage is mainly due to one sample reaching 44.0 % for a specific CAT activity = 15 µmol H_2O_2 mg protein^{-1} min^{-1} (Table 1).

Table 1. Intra- and inter-samples variabilities (in percentage of the mean) of the measurements of the quantity of protein extracted per wet weight and of the specific CAT activity of control biofilms extracted by trituration and ultrasonication and of exposed biofilms extracted by trituration, homogenization and homogenization followed by glass bead disruption.

Treatment	Extraction method	Protein extracted / wet weight (µg g^{-1})		Specific CAT activity (µmol H_2O_2 mg prot.$^{-1}$ min^{-1})	
		Intra-sample variability	Inter-samples variability	Intra-sample variability	Inter-samples variability
Control	Trituration	9.3 ± 4.1	55.5	9.2 ± 1.9	18.8
	Ultrasonication	2.6 ± 1.9	71.3	20.2 ± 11.9	2.7
Exposed to strong light intensity	Trituration	11.7 ± 7.1	28.7	4.2 ± 1.5	18.9
	Homogenization	3.6 ± 1.2	33.6	8.4 ± 3.4	65.8
	Homogenizaton + glass beads	3.4 ± 1.7	10.0	10.1 ± 4.4	26.8

Intra- and inter-samples variabilities refer to variabilities within analytical and experimental replicates, respectively. For intra-sample variability mean and standard error of experimental replicates are indicated.

3.1.3 Comparison of different extraction procedures

Analysis of results obtained by extraction by trituration showed no differences ($U = 3$, $\alpha = 0.1$) in CAT response between the two different sampling times (data not shown). Consequently, samples collected after 2h and after 8h were not differentiated when comparing the different extraction methods.

Extraction by ultrasonication was compared to extraction by trituration in control samples. Neither the total quantity of protein nor the protein concentration in samples extracted by ultrasonication were significantly different from the values obtained for samples extracted by trituration (Table 3). However, the protein quantity extracted per biomass was significantly higher in samples extracted by ultrasonication than in those extracted by trituration (Table 2 and 3). Though total CAT activity was not significantly different between samples extracted by one or the other method (Table 3), specific CAT activity was 2.8 times lower in samples extracted by ultrasonication than in those extracted by trituration (Table 2 and 3).

Extractions by homogenization and by homogenization followed by glass bead disruption were compared to extraction by trituration in samples exposed to strong light intensity. No significant differences were found between extraction by trituration and extraction by homogenization in terms of total quantity of protein extracted, quantity of protein extracted per wet weight, protein concentration and specific and total CAT activities (Table 3). The additional step of disruption with glass beads led to a significant increase in quantity of protein extracted per wet weight, in protein concentration and in specific and total CAT activities compared to extraction by trituration (Table 2 and 3).

Table 2. Quantity of protein extracted per wet weight, total protein extracted, protein concentration (conc.), total and specific CAT activity of control biofilms extracted by trituration and ultrasonication and of exposed biofilms extracted by trituration, homogenization and homogenization followed by glass bead disruption.

Treatment	Extraction method	Protein extracted / wet weight (µg g^{-1})	Total protein extracted (µg)	Protein conc. (µg mL^{-1})	Total CAT activity (µmol H_2O_2 min^{-1})	Specific CAT activity (µmol H_2O_2 mg $prot.^{-1}$ min^{-1})
Control	Trituration	230 ± 64	47 ± 15	115.2 ± 32.0	2.1 ± 0.7	46.1 ± 4.3
	Ultrasonication	1027 ± 423	112 ± 26	256.7 ± 105.7	1.7 ± 0.4	15.6 ± 0.2
Exposed to strong light intensity	Trituration	185 ± 31	56 ± 15	92.8 ± 15.4	1.4 ± 0.4	25.9 ± 2.8
	Homogenization	221 ± 43	65 ± 24	110.7 ± 21.5	3.8 ± 2.6	46.1 ± 17.5
	Homogenization + glass beads	307 ± 22	79 ± 14	153.5 ± 10.9	4.1 ± 0.1	53.8 ± 10.0

For all variables mean and standard error (SE) of experimental replicates are indicated.

Table 3. U values from Mann-Whitney test resulting from the comparison of the two-at-a-time extraction methods in terms of quantity of protein extracted per wet weight, total protein extracted, protein concentration (conc.), total and specific CAT activity of experimental replicates.

Methods compared	Protein extracted / wet weight	Total protein extracted	Protein conc.	Total CAT activity	Specific CAT activity
Trituration - Ultrasonication	**0**	1	2	6	**0**
Homogenization - Trituration	4	4	4	4	2
Homogenization + glass beads - Trituration	**0**	1	**0**	**0**	**0**
Homogenization + glass beads - Homogenization	1	2	1	2	2

Significant values are indicated in bold ($\alpha = 0.1$).

Regarding the quantity of protein extracted per wet weight, homogenization followed by glass bead disruption led to the lowest inter-samples variability followed by homogenization, trituration (mean of inter-samples variability for control and exposed samples: 42.1 ± 13.1 %) and ultrasonication. Regarding specific CAT activity measurements, the lowest inter-samples variability was observed in samples extracted by ultrasonication followed by those extracted by trituration (mean of inter-samples variability for control and exposed samples: 18.8 ± 0.1 %), homogenization followed by glass bead disruption and simple homogenization.

3.2 Field study

Inter-samples variabilities of specific CAT activity measurements from field samples were 26.4, 121.4 and 33.7 % for samples from S1, S2 and S3, respectively. The highest inter-samples variability (121.4 %) was observed for the lowest specific CAT activity measured (26.2 ± 22.5 μmol H_2O_2 min^{-1} mg $proteins^{-1}$ observed at S2). Inter-samples variabilities of quantity of protein extracted per wet weight were 33.0, 33.4 and 5.8 % for samples from S1, S2 and S3, respectively.

The three study sites presented high differences in both metal concentrations and specific CAT activity and smaller differences in photosynthetic efficiency (Table 4). Concentrations of Al, Cu and Pb were below detection limits at all sites. Pollution at S1 was mainly due to iron, and high concentrations of Zn, Ni and of the heavy metal cadmium were found at S2 and S3 (Table 4). Consequently CCU values were significantly different in all sites ($U = 0$ when comparing S1-S2, S1-S3 and S2-S3, $\alpha = 0.1$), S1 was characterized by an intermediate CCU value and S2 and S3 by high CCU values (Table 4). The total quantity of protein extracted, the quantity of protein extracted per wet weight as well as the specific and total CAT activities followed the inverse pattern, values of those variables in biofilm from S1 were significantly higher than those observed in S2 ($U = 0$, $\alpha = 0.1$) and S3 ($U = 0$, $\alpha = 0.1$; Table 4). Biofilms from S2 and S3 had similar total quantity of protein extracted and similar total and specific CAT activities but the quantity of protein extracted per wet weight was significantly higher in S3 than in S2 ($U = 0$, $\alpha = 0.1$). A significant negative correlation was found between CAT activity and CCU in water ($\rho = -0.86$, $p < 0.05$). Similar correlation was also found between specific CAT activity and Ni, Zn and Cd concentrations in water (for all: $\rho = -0.82$, $p < 0.05$), whereas the correlation between CAT and Fe concentration in water was not significant. Small, but significant differences ($U = 0$ when comparing S1-S2, S1-S3 and S2-S3, $\alpha = 0.1$) were found in photosynthetic efficiencies of biofilms from each site (Table 4). However, no significant correlations were found between photosynthetic efficiency and CCU or Ni, Zn or Cd concentrations in water ($p > 0.1$).

Table 4. Concentration of metals dissolved in water and their corresponding CCU as well as the quantity of protein extracted per wet weight, the total quantity of protein extracted and the total and specific CAT activities for each site.

		S1	S2	S3
Metals dissolved in water	Fe (µg L^{-1})	4532 ± 46.1	3303.7 ± 10.0	3018 ± 57.3
	Ni (µg L^{-1})	5 ± 0.3	29 ± 0.1	14.2 ± 0.8
	Zn (µg L^{-1})	b.d.l.	943.6 ± 17.6	555.5 ± 12.9
	Cd (µg L^{-1})	b.d.l.	13.9 ± 0.5	12.2 ± 0.4
	CCU	4.6 ± 0.0	25.1 ± 0.6	19.5 ± 0.5
Biofilm variables	Photosynthetic efficiency	0.329 ± 0.002	0.286 ± 0.003	0.359 ± 0.004
	Total protein extracted (µg)	635.2 ± 231.6	32.1 ± 5.6	40.7 ± 20.8
	Protein extracted / wet weight (µg g^{-1})	521.3 ± 99.2	149.3 ± 35.3	218.1 ± 8.9
	Total CAT activity (µmol H_2O_2 min^{-1})	119.4 ± 50.7	0.7 ± 0.6	1.6 ± 1.1
	Specific CAT activity (µmol H_2O_2 mg $prot.^{-1}$ min^{-1})	169.3 ± 25.8	26.2 ± 22.5	35.8 ± 8.5

b.d.l: below detection limit.

4. Discussion

4.1 Methods comparison in laboratory biofilms

The measurement of antioxidant enzyme activities in biofilms is an estimation of the capacity of the whole community to respond to oxidative stress. Previous studies highlighted the difficulties to extract antioxidant enzymes efficiently using glass tissue grinder since this instrument requires a high volume of sample (Bonnineau et al. 2010, Guasch et al. 2010). The methods described in the present study allowed the extraction and activity measurement of the antioxidant enzyme CAT from a smaller amount of

starting material corresponding to 2 cm^2 of laboratory biofilm and 4 cm^2 of field biofilms.

For all extraction methods, measurements of the quantity of protein extracted per wet weight and the CAT activity led to a low intra-sample variability indicating a good agreement between analytical replicates and so a good repeatability of these measurements. None of the extraction methods selected had a significant influence on intra-sample variability, showing a good repeatability of this measure in biofilms from laboratory. Nevertheless, a lower repeatability was observed for samples with low specific CAT activity. Therefore, it may be difficult to observe significant changes in samples for which specific CAT activity is lower than 15 µmol of H_2O_2 mg $protein^{-1}$ min^{-1}. Combining good extraction efficiency with a low detection limit is essential to detect a drastic decrease in CAT activity. For example, in this study, effects of strong light intensity on CAT activity were estimated with samples extracted by trituration. A strong photoinhibition led to significantly lower levels of CAT activity in exposed samples, close to detection limit, indicating that the use of a more efficient extraction method would allow more precision in this type of measurement. Moreover, this result confirms previous findings on CAT photoinhibition in algae (Lesser et al. 2006), showing the sensitivity of this enzyme to environmental factors.

First ultrasonication and trituration were compared. Extraction by ultrasonication led to a more efficient extraction of protein than extraction by trituration. Indeed, ultrasonication is a very vigorous process that is expected to completely disrupt cells and allow the release of cell walls and membrane-bound proteins (Cumming and Iceton 2001). Though ultrasonication may cause an increase in temperature and denature proteins (Janknegt et al. 2007), in the present study both methods preserve CAT integrity equally, as shown by the similar total CAT activity obtained in samples extracted by both methods. This result also showed that ultrasonication increased protein extraction but not CAT extraction, the proportion of CAT per protein was then smaller in samples extracted by ultrasonication than in

samples extracted by trituration. In addition, extraction by utrasonication required more extraction buffer than trituration, as the ultrasonic probe had to be covered by buffer during cell disruption, hence a similar protein concentration in samples extracted by ultrasonication and by trituration. Therefore, CAT concentration was lower in enzymatic extracts obtained by ultrasonication than in those obtained by trituration, as also shown by the lowest specific CAT activity. Without an additional concentration step, extraction by ultrasonication may not allow the detection of a CAT concentration as low as that detected in samples extracted by trituration. Moreover, ultrasonication was found to be a less reproducible method in terms of protein extracted per wet weight than trituration. Therefore, ultrasonication is not recommended for protein extraction of freshwater biofilms to perform CAT activity measurement.

Secondly, extraction by trituration, homogenization and homogenization followed by glass beads disruption were compared. The first two methods gave similar results in terms of protein extraction and CAT activities but trituration was a more reproducible extraction method than simple homogenization. The additional step of disruption by glass beads increased CAT extraction significantly, as both quantity of protein extracted per wet weight and total CAT activity were higher in samples extracted by homogenization and glass beads than in samples extracted by trituration. In addition, strength of trituration depends on the operator, while homogenization by a machine and especially cell disruption by glass beads is not operator-dependent; this difference may explain the higher reproducibility of extraction of protein by homogenization and glass beads than extraction by trituration. Moreover, this two-step extraction may be more appropriate for complex communities, such as biofilms. Homogenization may break preferentially the assemblage of biofilm and the biggest cells, while the extraction with glass beads may facilitate the disruption of cell membranes of smaller cells, such as bacteria or diatoms (Cumming and Iceton 2001).

Trituration was then found to be a more appropriate method than ultrasonication for CAT extraction in control biofilms whereas in exposed

communities extraction by homogenization followed by glass beads disruption was found to be better than extraction by trituration or simple homogenization. Though further experiments in control biofilms may be needed to confirm this last result. Homogenization followed by disruption with glass beads appears as the most complete method to extract CAT from freshwater biofilms. Further steps of optimization focusing on this method may improve even more CAT extraction by determining the optimal frequency and duration of time of beadbeater pulses, for instance. Extraction of other antioxidant enzymes (such as ascorbate peroxidase, glutathione reductase, glutathione-S-transferase, superoxide dismutase) by this method is likely to be successful as the same protocol is often used for extraction of different antioxidant enzymes.

4.2 Field study

The field study allowed the three sites to be characterized. Site S1 had moderate metal pollution while sites S2 and S3 were highly polluted according to CCU cut-off values defined by Guasch et al. (2009). Specific CAT activity was 4 to 6 times lower in the highly polluted sites than in the moderately polluted site while variations in photosynthetic efficiency were small between sites (-13 % at S2 and +10 % at S3 compared to S1). A strong correlation was found between CAT and CCU but photosynthetic efficiency was not correlated to CCU or to the concentration of any of the metals. In sites affected by chronic contamination tolerant communities are likely to be found (Soldo and Behra 2000). As photosynthesis is an essential process for community survival, photosynthetic efficiency is likely to be similar between different communities adapted to their environment, as shown in this field study.

Metals are expected to provoke oxidative stress in algae and periphyton (Pinto et al. 2003), so CAT was expected to participate in the response to chronic exposure to metals. High antioxidant enzyme activity was expected at S1 due to iron toxicity (Cassin et al. 2009). The decrease in CAT along the metal toxicity gradient found in this study was in accordance with Tripathi et al. (2006), who reported an induction of

antioxidant enzyme activities under mild oxidative stress and an inhibition under intense oxidative stress. The presence in S2 and S3 of Zn, Cd and of higher concentrations of Ni may be the cause of stronger oxidative stress leading to a decrease in CAT activity. Communities adapted to high metal concentrations presented a low CAT activity but might have developed other mechanisms to cope with oxidative stress induced by metals (pigments, thiols, etc.) or to limit metal toxicity (extracellular detoxification, reduced uptake, sequestration by phytochelatins, etc; Gaur and Rai 2001). The application to a broader range of concentrations in future studies will contribute to corroborate our observations. Nevertheless to confirm a cause-effect relationship the use of microcosms may be a pertinent alternative to a field study as this system allows a gradient of contamination to be simulated under controlled conditions (Clements and Newman 2002).

4.3 First steps towards the use of catalase as a biomarker of stress in biofilm communities

The use of complex communities in ecotoxicological studies allows a realistic approach, although biomarkers of such communities might lack precision (Clements and Newman 2002). The aim of this study was mainly to select an appropriate method for extraction of CAT from biofilm and to evaluate its feasibility and applicability in laboratory and field experiments. In the present study, in both laboratory and field, reproducibility of CAT activity measurements was good (26.8 % to 33.7 %) for almost all samples (except those from S1) and in the usual range found for other biomarkers in microcosms (Giddings and Eddlemon 1979). The measurement of CAT activity is, therefore, reproducible enough to allow CAT activity to be used as a biomarker in biofilms. Nevertheless, three to five replicates per group or sampling site are needed to be able to detect changes lower than 50% by ANOVA analysis.

The response of CAT from freshwater biofilms to usual factors inducing oxidative stress was also validated in the present study. In the field experiment, CAT activity was found to be a better biomarker of metal exposure than photosynthetic

efficiency. Though these examples illustrate the potential of CAT as an indicator of oxidative stress, they also showed that CAT is sensitive to both environmental (light) and chemical (metals) stressors. This limitation might be problematic in field studies where environmental parameters such as light or temperature are subject to variations. Therefore, further research may focus on natural variations of CAT in biofilms from different sites as well as laboratory experiments to determine the impact of specific factors on biofilm CAT activity. From this perspective, CAT in biofilm may not only be considered as an indicator of oxidative stress (as in the laboratory experiment), but also reflect biofilm communities strategies to respond to oxidative stress (as in the field study).

As a complement to this enzymatic approach, the study of variations in gene expression of CAT and other antioxidant enzymes may bring insight into the effects of toxicants on antioxidant enzymes regulation. Indeed it may allow to distinguish between changes at enzyme level (e.g. increase in activity but not in gene expression) and those at cell level (e.g. increase in activity and in gene expression). However the study of gene expression requires a priori knowledge of genetic sequences (Neumann and Galvez 2002). Hence, its application to a community containing mainly non-sequenced organisms, such as biofilms, would involve the resolution of numerous challenges.

5. Conclusion

After comparison of different extraction methods, the present study showed that extraction by homogenization followed by glass bead disruption was the most appropriate for CAT extraction from low amounts of biofilm samples. This method was successfully applied for the extraction of CAT from field biofilms where CAT was found to be strongly correlated with metal pollution. These preliminary results are encouraging and further applications in ecotoxicology will contribute to support the use of antioxidant activities as biomarkers of toxicity within fluvial biofilms.

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