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Abstract

Understanding the ecological status of aquatic ecosystems and the impact of anthropogenic contamination requires correlating exposure to toxicants with impact on biological communities. Several tools exist for assessing the ecotoxicity of substances, but there is still a

need for new tools that are ecologically relevant and easy to use. We have developed a protocol based on the substrate-induced respiration of a river biofilm community, using the MicroResp™ technique, in a pollution-induced community tolerance approach. The results show that MicroResp™ can be used in bioassays to assess the toxicity toward biofilm communities of a wide range of metals (Cu, Zn, Cd, Ag, Ni, Fe, Co, Al and As). Moreover, a community-level physiological profile based on the mineralization of different carbon substrates was established. Finally, the utility of MicroResp™ was confirmed in an in-situ study showing gradient of tolerance to copper correlated to a contamination gradient of this metal in a small river.

Capsule: A modified MicroResp™ technique as a tool for measuring induced tolerance to heavy metals of microbial biofilm community.

Keywords: river biofilm; pollution-induced community tolerance; substrate induced respiration; heavy metals; MicroResp™.

I. Introduction

In a setting of agricultural land use, small lotic ecosystems (Strahler order below 3) are very sensitive to pollution because of generally low dilution and contiguous contamination sources (spraying of pesticides, run off…) (Dorigo et al., 2003). In these ecosystems, biofilms (attached microbial communities of autotrophic and heterotrophic, eukaryotic and prokaryotic populations) play a fundamental role in the aquatic trophic web and geochemical cycles (Battin et al, 2003). In agricultural watershed rivers, biofilms are exposed directly to toxicants that run off from fields, and their structure and function can be affected. The vast diversity of microbial species and the multitude of biological and physico-chemical processes induce a large structural and functional complexity into the matrix biofilm. Also, species composing the biofilm may have ranging sensitivities and responses towards various anthropogenic pressures (Barranguet et al., 2003). During exposure to toxic agents such as heavy metals, the most sensitive organisms may be overtaken by more resistant or more tolerant ones. Thus the entire community may be restructured, present physiological alterations and come finally to display an overall increase in tolerance to the toxicant. This process underlies the concept of pollution-induced community tolerance (PICT), developed by Blanck et al. (1988) as an ecotoxicological tool to assess xenobiotic impact at the community level.

Diverse approaches to examining the tolerance or resistance of a bacterial community to heavy metals have been developed. For example, Díaz-Raviña et al. (2007) investigated vineyard soil copper contamination inducing bacterial tolerance with growth measurements using thymidine (Tdr) and leucine (Leu) incorporation techniques. In aquatic systems Tdr or Leu incorporation techniques have also been used to assess the tolerance of bacterial communities in biofilm and sediments (Paulsson et al., 2000; Lesley and Grant, 2007). Despite good results, these techniques remain difficult to implement and are not possible in all laboratories as they use radio-labelled elements. Biolog^{TM} is a conventional approach applied first to obtain catabolic fingerprints of the bacterial communities or a community-

level physiological profile (CLPP), and more recently to assess tolerance to metals of the bacterial communities in soil and aquatic biofilms (Boivin et al., 2006; Stefanowicz et al., 2009). However, this microplate technique, based on the analysis of a bacterial community's use of several carbon sources, has major drawbacks: incomplete assessment of the whole microbial community, a long incubation time inducing bacterial selection and growth (Preston-Mafham et al., 2002) and the use of artificial culture media with buffer that may have artificial significant effects on the speciation and in consequence on the bioavailability and the toxicity of toxicants (Barranguet et al. 2003). A respirometric technique based on the analysis of the substrate-induced respiration (SIR) response (using gas chromatography analysis) was also investigated with a multiple carbon-source substrate for CLPP (Garland and Mills, 1991), and in a few studies, to test microbial tolerance to heavy metals at the community level in soils (Witter et al., 2000; Rajapaksha et al., 2004). More recently this approach was used to assess induced tolerance to copper of bacterial communities in river biofilms (Tlili et al., 2010). However, the method is still tedious, especially when assaying many microbial samples simultaneously in a multitude of bottles that have to be processed separately to measure the amount of released CO_2 (Chapman et al., 2007). Miniaturized systems offer the considerable advantage of allowing sample and replicate multiplication. In soil studies, Campbell et al. (2003) developed the MicroResp™ technique, which is an alternative method that combines the advantages of the Biolog™ technique, using the microplate system, and of the SIR approach with ability to measure CO_2 production during short-term incubation from a whole soil microbial community.

We hypothesized that the use of this new microrespirometric technique could be modified and applied to perform ecotoxicological bioassays on river biofilms for metal tolerance comparison purposes. The objectives of our study were thus (i) to test the MicroResp™ technique for a metal-contamination assessment in an aquatic ecosystem, using biofilms as a biological model in a PICT approach, (ii) to take a catabolic fingerprint of the heterotrophic

biofilm community, and (iii) to implement an in-situ PICT application of MicroResp™ in a copper contamination context.

II. Materials and methods

1. In-situ biofilm

The biofilm used in our study was taken from the River Morcille, located in the Beaujolais vineyard area. The Morcille is a small first-order stream (7 km long) under strong agricultural pressure, essentially from vineyards, which occupy about 80% of its 8.5 km^2 catchment area. The Morcille watershed is part of the Long-Term Ecological Research Rhône Basin (ZABR). Regular physico-chemical surveys (hydrology, nutrients, pesticides and metals) and biological monitoring have been conducted on this site (Dorigo et al., 2007; Rabiet et al., 2008; Morin et al., 2010; Montuelle et al., 2010). Three sampling sites were selected along a gradient of increasing percentage contribution of vineyards to the catchment area: Saint-Joseph (vineyard percentage cover 11%), Les Versauds (53%) and Saint-Ennemond (65%) (see Table. 1 for physico-chemical details). Several annual surveys were performed to characterize metal contamination. In 2009 (n = 43 samples), Saint-Joseph was the least polluted site with a mean 0.51 ± 0.02 $\mu g.L^{-1}$ of Cu, 1.13 ± 0.21 $\mu g.L^{-1}$ of Zn and 1.94 ± 0.46 $\mu g.L^{-1}$ of As. By contrast, the two other sites studied were considered polluted: Les Versauds was characterized by a mean 2.05 ± 0.25 $\mu g.L^{-1}$ of Cu, 0.83 ± 0.42 $\mu g.L^{-1}$ of Zn and 11.5 ± 0.46 $\mu g.L^{-1}$ of As, and Saint-Ennemond by a mean 3.93 ± 0.77 $\mu g.L^{-1}$ of Cu, 7.05 ± 3.56 $\mu g.L^{-1}$ of Zn and 9.26 ± 4.99 $\mu g.L^{-1}$ of As. Other metals including Cd, Ni, Fe or Al were also detected at low levels (means near limits of quantification) and there was no gradient of contamination from upstream to downstream.

Table.1

2. Biofilm microcosm

During the different experiments with the MicroResp™, a biofilm was grown on artificial substrates (microscope slides previously cleaned in a dilute nitric acid bath and rinsed with milli-Q water before use) in laboratory microcosms under controlled conditions. Fifteen-litter aquariums were filled with river water from the reference site (Saint-Joseph), which had been filtered through a 50 µm mesh to remove most of the grazers (water was renewed once a week). The water was kept at a temperature of 18 ± 2 °C, and exposed to a light intensity of 260 µmol $m^{-2}s^{-1}$, with a light/dark regime of 18:6 h. Three-week-old biofilms were used for the MicroResp™ assays (Tlili et al., 2008).

3. The MicroResp™ procedure

MicroResp™ system has been described in detail by Campbell et al. (2003), but only a few ecotoxicological applications of the MicroResp™ technique have been investigated on soils (Kaufmann et al. 2006) and none on aquatic microbial communities. Here we present an adaptation of the procedure of Campbell et al. (2003) to aquatic biofilm suspensions and toxicological bioassays. MicroResp™ is a colorimetric method based on the colour change of a pH indicator dye caused by the release of CO_2 by heterotrophic communities. The system consists of two microplates (96 wells) placed face to face. One of these is a deep-well microplate (1.2 mL capacity, 96-deep-well microplate, NUNC) in which each well contains the microbiological sample (we used a 500 µL biofilm suspension obtained by scraping biofilm off glass substrata using a polypropylene spatula and suspending it in 0.2 µm of Nuclepore-filtered water from the reference site) with the carbon source (30 µL per well, 6.2 mg of C per well). To this deep-well microplate we added the toxicant (50 µL per well). The second microplate contained the detection gel (cresol red dye (12.5 ppm), potassium chloride (150 mM) and sodium bicarbonate (2.5 mM) set in a 1% gel of noble agar (final volume 150 µL per well)). The two microplates were sealed together with a silicone seal, with interconnecting holes between the corresponding wells. The assembly was clamped together and the system was incubated in the dark (to avoid any photosynthesis interference with CO_2

release) at room temperature. CO_2-trap absorbance was measured at 590 nm (Biotek Synergy HT spectrophotometer) immediately before sealing to the deep-well plate (coefficient of variation below 5%), and after incubation. Quantities of CO_2 produced by the microbial samples were calculated using a calibration curve of absorbance values versus CO_2 quantity measured by gas chromatography (MTI 200 microcatharometer). Results were then expressed as µg $CO_2.mg^{-1}$ of sample.h^{-1}. In our biofilm study, the sample was considered as organic matter (ash-free dry weight, AFDW) of the biofilm suspension tested, and measured using the protocol described by Tlili et al. (2008). Briefly, three aliquots of each homogenized biofilm suspension (2 mL) were filtered through individual 25 mm CF/C Whatman glass fibre filters (1.2 µm pore size). Total dry matter was measured by weighing the filters after drying at 105°C for 24 h. The filters were then combusted in an oven at 480°C (Nabertherm P320) for 1 h, and reweighed to calculate mineral matter. AFDW was calculated by subtracting mineral matter from total dry matter.

4. Development of the MicroResp™ protocol

Table.2

4.1. Incubation time

Campbell et al. (2003) propose an incubation time of 6 h with soil samples. However, in our case, we have to take into account the rate of CO_2 diffusion at the air-water interface, which may be low. To ensure a relevant measure in the well airspace of the CO_2 released from the sample (the biofilm), we tested a range of incubation times: 4, 6, 8, and 15 h. Individual MicroResp™ microplates were used for each incubation time. Eight deep wells of each microplate were filled with 500 µL of biofilm suspension with 50 µL of milli-Q water and 30 µL of D-glucose (Prolabo) (the remaining wells of the microplate were filled with 580 µL of milli-Q water). Quantities of released CO_2 were measured at each incubation period, as described in Section 3.

4.2. Pre-incubation test with toxicant

To enable the toxicant to penetrate into the cells and to cope with artefacts causing a chemical release of CO_2 (Lindsay, 1979), a metal pre-exposure period was necessary before adding the carbon substrate (glucose) and starting the incubation. The different tested times of pre-exposure to Cu were 0, 0.25, 0.5, 1, 2, 3, 4 and 6 h.

4.3. Short-term bioassays

To validate the utility of the MicroResp™ method for short-term bioassays, using biofilms, a wide range of heavy metals was tested: $CuSO_4$ (Merck KgaA; purity≥99.0%;), $ZnSO_4$ (Sigma-Aldrich GmbH), $CdN_2O_6.7H_2O$ (Fluka Sigma-Aldrich; purity≥99.0%), $AgNO_3$ (CARLO ERBA reagenti; purity≥99.8%), $N_2NiO_6.6H_2O$ (Fluka Sigma-Aldrich Chemie GmbH; purity≥97.0%), $FeSO_4.7H_2O$ (Sigma Chemical co.; purity≥99.0%), $CoN_2O_6.6H_2O$ (Fluka Sigma-Aldrich Chemie GmbH; purity≥98.0%), $AlN_3O_9.9H_2O$ (Merck KgaA; purity≥98.5%) and $Na_2HAsO_4.7H_2O$ (Sigma-Aldrich GmbH; purity≥98.0%). Briefly, stock solutions containing 200 mM of each metal were prepared in water and stored at 4 °C, before dilution in the test wells. A semi-logarithmic series of concentrations was freshly prepared by serial dilution of the stock solutions in 0.2 µm-filtered river water. Final nominal test concentrations in the deep wells ranged from 0.5 µM to 17.2 mM (4 blanks and 4 replicates for each of the 9 concentrations). The carbon substrate used for all the SIR toxicological bioassays was D-glucose. Quantities of released CO_2 were measured for each bioassay as described in Section 3. Dose-response curves were plotted using CO_2 values produced by biofilm at each metal concentration as a percentage of CO_2 produced by a control biofilm (without toxicant).

5. Application of the microrespirometric method to a field situation

5.1. PICT approach (short-term bioassays)

The relevance of the MicroResp™ technique in a PICT approach was tested with biofilms sampled from the River Morcille. Induced tolerances to Cu, Cd and Ag of the heterotrophic biofilm communities were then tested by toxicological bioassay using the MicroResp™

method. Briefly, biofilms were scraped from stones (500 cm² per sample) collected from the three sampling sites described previously (St. Joseph (J), Les Versauds (V) and Saint-Ennemond (E)), and homogenized in 150 mL of 0.22 µm-filtered water from the reference site (J). Biofilm suspensions were placed directly in the deep well (500 µL) and 50 µL of each tested metal (Cu, Cd or Ag) was added separately. After pre-incubation 30 µL of glucose was added to each of the deep wells, and the MicroResp™ system was assembled as described previously and incubated in the dark at room temperature (23 ± 1 °C). After incubation, the absorbance of the detection microplate was measured at 590 nm (Biotek Synergy HT spectrophotometer), the amounts of released CO_2 were calculated and the results were expressed in µg $CO_2.mg^{-1}AFDW.h^{-1}$. Dose-response curves were plotted using CO_2 values produced by the biofilm at each metal concentration as a percentage of the CO_2 produced by control biofilms (without toxicant). The EC_{50} values were calculated for each metal and for each site.

5.2. Catabolic diversity fingerprint

With a view to establishing a CLPP of the heterotrophic biofilm compartment, a multiple carbon-source substrate-induced respiration approach (using the MicroResp™ technique) was used. The different carbon substrates used were: D-glucose (Prolabo), D-fructose (Sigma chemical Co.), D-sucrose (Fluka biochemica), D-ribose (Prolabo), D-galactose (Prolabo), D-maltose (Prolabo), L-arginine (Sigma Chimie Fluka), glycine (Sigma Aldrich Chemie), L-lysine hydrochloride (Sigma Chimie, Fluka), L-glutamic acid (Sigma Aldrich Chemie) and citric acid anhydre (Sigma Aldrich Chemie). Stock solutions containing 120 $mg.mL^{-1}$ of each substrate were prepared in 0.22 µm-filtered Milli Q water and stored at 4 °C, until used. The pH of each solution was adjusted to river pH (7) to prevent any substrate-pH effects on microbial communities and minimize chemical artefacts due to carbonate-derived CO_2. Final concentration was 6.2 mg of C per mL in each deep well. The results were expressed in µg

CO_2.mg^{-1}AFDW.h^{-1}. In addition to the SIR measurements, the basal respiration (without substrate addition) was measured for each biofilm.

6. Biofilm DNA extraction, amplification and automated ribosomal intergenic spacer analysis (ARISA)

To characterize the diversity of the river biofilm bacterial communities, we used the ARISA molecular fingerprint technique. Three replicates of 2 mL of each biofilm suspension were centrifuged at 14,000*g* for 30 minutes, and the supernatant was discarded and pellet stored at -80 °C before extraction. Nucleic acid extraction was performed on biofilm pellets using the FAST DNA kit (QBIOgene, Illkirch, France) according to the manufacturer's instructions. The PCR conditions and the PCR template preparation for the ARISA conditions were those described by Ranjard et al. (2003). The primers used were S-D-Bact-1522-b-S-20 (3′end of 16S genes) and L-D-Bact-132-a-A-18 (5′end of 23S genes) (Ranajard et al., 2003).

7. Data treatment

EC_{50} values were calculated using the Regtox model (E. Vindimian, http://eric.vindimian.9online.fr/). The CLPP data sets (normalized by the basal respiration values) were subjected to principal component analysis (PCA) (XLSTAT Software Package. 2009 version). The significant level was set to 5% for all the statistical tests with four replicates for each parameter. Bacterial ARISA profiles were compared with regards to the presence or absence of bands, using the Pearson similarity index. Matrices were used to perform Ward's method of hierarchical cluster analysis (HCA) using the XLSTAT Software Package (2009 version).

III. Results and discussion

1. MicroResp™ protocol applied to ecotoxicological bioassays on microbial biofilms

In toxicological bioassays, adding a mineralizable carbon source overcomes a problem that arises when measuring basal respiration, namely mineralization of unknown native organic

matter that may be different between sampling stations. Glucose mineralization activity was detected by our MicroResp™ modified design within 8 h incubation time, and net CO_2 production was approximately linear between 8 h and 15 h incubation time, with respiration rates of 160.0 ± 14.9 and 160.6 ± 12.1 µg CO2.mg^{-1}AFDW.h^{-1} for 8 h and 15 h incubation time respectively. In addition, analysis of the bacterial community profiles (ARISA) of each of the four incubation times tested showed no change in the diversity of this community (data not shown). Incubation for 8 h would have sufficed, but for practical reasons we opted to conduct our bioassays with an incubation time of 15 h (overnight).

During preliminary bioassays with Cu, no dose-response curves were obtained (data not shown). The quantities of measured CO_2 corresponding to the well containing the most concentrated Cu solutions were high. The pH measurement of these biofilm solutions showed that the addition of large amounts of Cu to the biofilm suspensions induced a very rapid and marked pH decrease, linked to an abundant release of CO_2, which could not be of biological origin but was probably due to chemical reactions. Oren and Steinberger (2008) suggested that dissolution of $CaCO_3$ (contained in the biological samples, in their case soil) may follow pH decline, and be accompanied by abiotic CO_2 evolution that may be mistakenly taken for respired CO_2. Fig. 1 shows that 3 h of copper pre-incubation is needed to obtain a dose-response curve and a percentage SIR inhibition below 20% (19.01 ± 0.1%) with the maximum copper concentration (17.2 × 10^2 µM). This dose-response curve did not change with biofilms pre-incubated for longer times (4 h and 6 h). These results suggest that this chemical artefact has ceased after 3 h. We therefore opted for this time of 3 h pre-incubation with copper.

Fig.1.

Finally, the protocol used for short-term bioassays can be summarized as follows: the biofilm suspension was distributed in deep wells (500 µL per well), to which we added 50 µL of the metal solution (increasing nominal concentrations). The microplate was then pre-incubated in the dark at room temperature for 3 h, 30 µL of the glucose solution (120 mg.mL^{-1}) was added

to each well and the detection microplate was positioned. The system was sealed and incubated for 15 h in the dark (without photosynthetic activity, which could induce an increase of the pH and interfere with the CO_2 release) and at room temperature (23 ± 1 °C). CO_2-trap absorbance was measured at 590 nm (or better at 570 nm, Rowell, 1995) immediately before sealing to the deep well plate, and after 15 h incubation.

The MicroResp™ technique enabled us to plot dose-response curves with all the tested metals. The EC_{50} obtained with the different tested metals in short-term bioassays are shown in Table.3. Compared with the other metals, silver seems to be the most toxic metal to the SIR (EC_{50} = 3.96 µM). Aluminium (EC_{50} = 2317.86 µM) and cobalt (EC_{50} = 6170.63 µM) were the least toxic, while Zn, Cu and Cd had similar intermediate toxicities. However, in our study some of the metal salts used included a nitrogen source (e.g. $AgNO_3$; $N_2NiO_6.6H_2O$ or $CoN_2O_6.6H_2O$) that could interfere with the respiration measurements and so bias the EC_{50} values. A second control using $NaNO_3$ in addition to glucose might be a good option to overcome this interference. Witter et al., (2000) obtained similar results when testing soil bacteria tolerance (based on SIR) to these three metals (e.g. EC_{50} Zn value of about 250 ppm). EC_{50} values calculated for the percentage of 95 substrates used completely within 8 days of incubation in Cu (0–300 mM)-amended BIOLOG GN plates inoculated with a river biofilm bacteria consortium were in the range 10–30 µM (Barranguet et al. 2003). Sensitivities obtained with other bioassays such as the well-known Microtox® (based on bioluminescence reduction of the marine bacterium *Vibrio fischeri*) are also comparable to our range of EC_{50} values (e.g. EC_{50}(5 mn) = 941 µM $CdCl_2$ and 168 µM $CuCl_2$ and EC_{50}(15mn) = 421 µM $CdCl_2$ and 49 µM $CuCl_2$, in Macken et al. 2009). Therefore, our dose-response curves and EC_{50} obtained with the SIR, related to heterotrophic organisms, confirm that the MicroResp™ technique could be a useful method to classify heavy metals according to their toxicity toward river biofilms.

Table.4.

2. Application of the microrespirometry method to a field situation

2.1. Copper-induced tolerance

Cu EC_{50} values were estimated to evaluate tolerance to this pollutant of the biofilm communities sampled at three sites along the River Morcille. EC_{50} values increased upstream to downstream, ranging from 48.5 to 179.4 µM copper (Table.3, Fig.2). The most Cu-sensitive communities were from the upstream site "Saint-Joseph" (**J**), while the most Cu-tolerant communities were downstream at "Saint-Ennemond" (**E**). The measured sensitivity at the intermediate site "Les Versauds" (**V**) was closer to **E** than to **J**. Cu concentrations in the water from the three sampling sites, collected in the same period as our study, and the EC_{50} values measured were closely correlated (n = 4, Pearson R^2 = 0.72, p = 0.0004). Also, diversity analysis by ARISA of bacterial communities showed a differentiation of **J**, **E** and **V**. Bacterial diversity from the intermediate site **V** was more closely similar to the most contaminated site **E** than the pristine area **J** (Fig.3). These results agree with previous studies (Boivin et al., 2006; Dorigo et al., 2007) and are congruent with the predictions of the PICT concept and validation (Blanck, 2002), suggesting that Cu constitutes a selective pressure, at least on the bacterial and heterotrophic portion of the Morcille biofilms, producing changes in species composition, catabolic structure (see paragraph 2.3) and Cu substrate-induced respiration tolerance. However, given the presence of gradient contaminations with other metals (As and Zn) from the Morcille upstream to downstream, these differences in bacterial structure cannot be attributed to Cu alone.

On the other hand, environmental factors such as light, current velocity, temperature or nutrient content could affect toxicant bioavailibility and the biofilm tolerance to these toxicants. During long-term exposure, for example, Guasch et al. (2004) and Tlili et al. (2010) showed that phosphorus supply caused an increased induced tolerance of biofilms to copper. Also, Guasch et al. (2002) evaluated the effects of chronic copper exposure on natural periphyton in a non-polluted calcareous river, and they concluded that the water pH in the river was important in affecting the bioavailability of the metals and therefore their toxicity towards the biological community. It is therefore necessary to have not only a reference site that is globally comparable to the study site, but also a sampling strategy (spatial and temporal) that minimizes the interference of environmental factors in the detection of PICT (Dorigo et al., 2009). Care must also be taken in a PICT approach to ascertain that bioassays

are controlled and standardized, and that different physicochemical parameters are homogeneous and similar during short-term exposure of the tested communities. Blanck (2002) stipulated that bioavailability may interfere in the PICT detection as a confounding factor, for example when the waters used in the short-term tests are different. This could then affect the EC_X values obtained and finally the PICT signal may represent a bioavailability gradient. However, this remark concerns a regional survey (Blanck et al., 2003) that compared very different sampling sites (from different rivers and countries) in the context of bioassays that used the filtered natural water from each sampling area. This was not the case in our study, where we used the same filtered water of the upstream sampling area **J** (pH values in the range 7.2–8) for periphytic suspensions and toxic solutions of all bioassays and in the context of a small river basin. In addition, in a previous study we measured pH in the biofilm-river suspension (using water from site **J**), glucose and copper added at the beginning and end of the short-term incubation, and observed no pH changes during the bioassays despite demonstrating an induced tolerance to copper based on the substrate-induced respiration as a functional parameter (measured by gas chromatography, data not shown). Rusk et al. (2004) investigated tolerance of soil biological nitrification to metals using sterilized, metal-treated soils from the reference area reinoculated with a similar soil containing the microbial communities to be tested. Our approach using filtered water from a reference area (upstream) to perform the PICT bioassays, in which we suspended periphyton scraped from the different investigated sites, is comparable to the approach of Rusk et al. (2004), who wanted to exclude the potentially confounding effects of variations in metal bioavailability in their tolerance bioassays. We can conclude that the bioassays conducted during our study were performed under homogeneous standardized conditions and minimized variations in metal bioavailability among the microbial samples tested.

Table.4. Fig.2. Fig.3.

In previous studies, a respirometric technique based on the analysis of the substrate-induced respiration (SIR) response was developed to test the microbial tolerance to toxicants at the community level in soil ecosystems (Witter *et* al., 2000). These studies demonstrated that microbial tolerance to metals increased as soil metal pollutant concentrations increased. However, there are few studies on the toxicity assessment of contaminants in aquatic ecosystems using the SIR to assess the induced tolerance to metals of heterotrophic biofilm communities. Dorigo et al. (2010), investigated the effects of Cu on heterotrophic biofilm communities collected from the same sampling sites (**J**; **V** and **E**), using the SIR measured by gas chromatography and the 16S rRNA gene DGGE method to highlight induced tolerance to Cu and bacterial diversity respectively. Like ours, her results strongly suggested Cu contamination-driven changes in biofilm community structure and in the tolerance to Cu of the bacterial community, confirming the PICT hypothesis.

This first biofilm ecotoxicological bioassays using MicroResp^{TM} applied in a river contamination assessment context, highlights the efficiency of this technique for measuring the induced tolerance to metals of the heterotrophic biofilm community based on SIR.

2.2. Co-tolerance assessment

Co-tolerance may occur when communities that have been exposed to one toxicant, but not to another, become tolerant to both toxicants. Occurrence of co-tolerance depends on the means of conferring tolerance and on the tolerance mechanisms (Blanck et al., 1988). Tolerance to Ag and Cd was measured for biofilms from the three sampling sites (**J**, **V** and **E**) (Table.3). In contrast to results for copper, no significant changes in tolerance to Ag or Cd were observed from upstream to downstream sites. These results are congruent with Ag and Cd concentrations measured in water from **J**, **V** and **E** sites, which were constantly below the limits of quantification. Therefore, these SIR measurements with MicroResp^{TM} did not reveal any co-tolerance between Cu and Ag or Cd. Some studies have reported a co-tolerance of biofilm communities between copper and some metals such as Zn or Ni (Gustavson and

Wängberg, 1995; Ivorra et al., 1999). Soldo and Behra (2000) have shown a strong co-tolerance to silver in autotrophic biofilm communities exposed to 5µM of copper. In contrast to our study, all these co-tolerance measurements were based on photosynthetic activity, and therefore especially targeted the phototrophic biofilm compartment. Detection of co-tolerance implies difficulty attributing a tolerance shift to the presence of a particular toxicant in the environment. Our results and those obtained in the above-cited studies show that depending on the kind of targeted activities and associated communities (phototrophic or heterotrophic), co-tolerance linked to specific modes of action of toxicants and detoxification processes (Soldo and Behra, 2000; Knauer et al., 2010) can be variable. MicroResp^{TM} use, in addition to other tolerance measurements based on other activities in the context of co-tolerance studies, could thus offer a complementary approach to investigating mechanisms of tolerance.

2.3. Community-level physiological profiles

Principal component analysis (PCA) was applied to the data set obtained with the various carbon sources, normalized by the basal respiration values for each site (Fig.4). The first two axes of the PCA accounted for more than 80% of the variability. Pristine area **J** and downstream area **E** were separated from the intermediate area **V** on the first axis F1, while the second axis F2 separated area **J** from area **E** (left panel). Biofilms from **V** were characterized by a higher mineralization of the glutamic acid and citric acid substrate and biofilms from **J** and **E** were characterized by a higher mineralization rate of glucose substrate (right panel). Thus multivariate analysis showed a clear discrimination between the biofilms from the different sampling sites. Several soil studies investigated functional diversity with CLPP measurements and showed the utility of the MicroResp^{TM} method to discriminate different soils or soils submitted to different pressures (Campbell et al., 2003, 2008; Oren and Stenberger, 2008). Applied to an aquatic environment, MicroResp^{TM} seems also to be a useful tool for assessing changes in the functional diversity of the microbial community (CLPPs) in biofilms.

Fig.4.

VI. Conclusion and perspectives

In the global context of assessing the impact of pollutants on the diversity and functioning of microbial communities in aquatic ecosystems, a range of diversified indicators for diversified functions and communities is needed (Clements and Rohr, 2009). Among such indicators, the PICT approach, which offers a good tool for assessing an ecosystem's history of exposure to pollution at the community level, and the establishment of catabolic fingerprint profiles of microbial communities, are now applied in diverse ecosystems (Bérard et al., 2002; Boivin et al., 2006; Kaufmann et al., 2006). However, this study is the first to investigate micro-SIR in a contaminated aquatic system as a tool for measuring induced tolerance in a microbial community and establishing catabolic physiological profiles on the whole heterotrophic biofilm. The purpose of this methodological study was not to make a direct translation of the EC_{50} obtained in MicroResp™ plates to the situation of the biofilms exposed in the river, but to propose an easy short-term bioassay based on catabolism to detect relative differences in metal tolerance between long-term exposed communities that were sampled (complementary to investigations of their taxonomic composition for PICT methodology). Our results show that the MicroResp™ technique offers a convenient, rapid and sensitive method for assessing metal contamination of aquatic ecosystems. Like for soil microbial micro-SIR measurements, for results to be fully transportable between studies, this method would require inter-laboratory calibration (Creamer et al., 2009). A modified MicroResp™ system could be developed, using microplates with larger wells than those currently used. The method could thus be applied with an in-place biofilm (grown on individual artificial substrates) to minimize disturbances of the microbial communities and be suitable for similar samples of periphyton, and for other measurements of additional activities such as photosynthesis (Dorigo and Leboulanger, 2001).

Acknowledgments

The authors thank G. Sevenier (UMR EMMAH, INRA-UAPV, France) for technical advice on the MicroResp™ technique, M. Coquery, J. Gahou for metal analysis, B. Motte (UR MAEP, CEMAGREF-Lyon, France) for field sampling and ATT for editing the English-language version of the manuscript. We thank Onema (French National Agency for Water and Aquatic Ecosystems) for financial support (Program 26 "Assessment and Remediation of Pesticide Effects").

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Table.1. Means (± standard deviation, n = 43) of selected chemical parameters (pH; cond.: conductivity [µS.cm^{-1}]; DOC [mg.L^{-1}]; NH_4^+ [mg.L^{-1}]; NO_2^- [mg.L^{-1}]; NO_3^- [mg.L^{-1}] and PO_4^{3-} [mg.L^{-1}]) obtained from the three sampling areas (J: Saint-Joseph; V: Les Versauds and E: Saint-Ennemond) over two years (2009 and 2010).

Site	pH	cond	DOC	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}
J	7.27 ± 0.29	161.72 ± 17.65	2.67 ± 0.88	0.04 ± 0.03	0.02 ± 0.01	6.82 ± 1.28	0.07 ± 0.03
V	7.41 ± 0.27	201.33 ± 18.57	3.28 ± 0.91	0.05 ± 0.04	0.03 ± 0.01	7.58 ± 3.03	0.23 ± 0.13
E	7.51 ± 0.24	233.61 ± 33.52	4.52 ± 1.82	0.08 ± 0.04	0.06 ± 0.06	7.33 ± 2.69	0.28 ± 0.17

610 Table. 2. Chronological summary of the different experiments and steps performed during the
611 study
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	Step No.	Biofilm used
Experiment 1 **Development of the MicroResp™ protocol, applied to aquatic ecosystems and using river biofilms.**	1. Incubation time To ensure a relevant measure in the well airspace of the CO_2 released from the sample. 2. Pre-incubation test with toxicant To cope with artefacts causing a chemical release of CO_2. 3. Short-term bioassays To validate the utility of the MicroResp™ method for short-term bioassays with different metals.	Cultivated in laboratory microcosms under controlled conditions
Experiment 2 **Application of the microrespirometric method to a field situation**	1. PICT approach (short-term bioassays) To validate the relevance of the MicroResp™ technique in a PICT approach, by testing copper, silver and cadmium. 2. Catabolic diversity fingerprint To establish a CLPP of the heterotrophic biofilm compartment, using a multiple carbon-source substrate. 3. Molecular biology analysis To characterize the diversity of the river biofilm bacterial communities, using the ARISA molecular fingerprint technique.	Collected from the three sampling sites in the Morcille River.

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Table. 3. Sensitivity (expressed as EC_{50} in µM) of the heterotrophic biofilm community to different tested metals in short-term bioassays using MicroResp™. C.I. confidence interval ($n = 4$; $\alpha = 0.5$)

Tested metal	EC_{50} µM	C.I.
Silver	3.96	2.60 – 6.27
Zinc	82.41	15.77 – 323.18
Copper	156.45	122.27 – 196.44
Cadmium	194.95	110.99 – 336.18
Arsenic	244.46	178.52 – 348.43
Nickel	403.53	87.14 – 1184.89
Iron	772.88	392.85 – 1532.95
Aluminium	2317.86	1515.83 – 3731.99
Cobalt	6170.63	4270.30 – 8519.66

Table. 4. EC_{50} values (in µM of copper, silver or cadmium) for the three sampling sites (J: Saint-Joseph; V: Les Versauds; E: Saint-Ennemond). C.I. confidence interval ($n = 4$; $\alpha = 0.5$).

Tested metal	Site	EC_{50} µM	C.I.
Copper	J	48.52	41.02 - 59.13
	V	172.37	133.50 - 227.91
	E	179.42	117.11 - 266.18
Silver	J	31.99	25.99 - 40.67
	V	22.39	17.25 - 31.37
	E	25.52	19.22 - 36.01
Cadmium	J	82.48	65.59 - 103.61
	V	111.15	91.04 - 142.36
	E	82.48	65.59 - 103.61

Fig. 1. Respiration values (expressed as a percentage of control) after short-term bioassay with different pre-incubation periods (▲ 0; ◆ 0.25; ● 0.5; ■ 1; ∗ 2; ◇ 3 ; □ 4 and △ 6 h) before addition of the carbon substrate (glucose). Error bars represent standard deviations (n = 4).

Fig. 2. Copper tolerance of heterotrophic communities at three sampling areas along a copper contamination gradient in the River Morcille. Symbols for each area (◆: Saint-Joseph; ■: Les Versauds; ▲: Saint-Ennemond), represent average SIR expressed as a percentage of the corresponding controls and obtained during the MicroResp™ short-term bioassay. Dose-response curves (◇: Saint-Joseph; □: Les Versauds; △: Saint-Ennemond) represent the Hill equation model applied to the same SIR measurements. Parameters were calculated by nonlinear regression and statistics (four replicates) were obtained using a Bootstrap-Monte-Carlo simulation (from Regtox model).

Fig 3. Cluster analysis (hierarchical ascendant classification based on Pearson correlation coefficient) of the bacterial community (ARISA analysis of PCR amplified IGS gene fragments) from three sampling areas (J: Saint-Joseph; V: Les Versauds; E: Saint-Ennemond) on the River Morcille (three replicates per sampling area).

Fig 4. Principal component analysis (PCA) of community-level physiological profiles (CLPP) of biofilms measured with the MicroResp™ method and normalized to basal respiration, from three sampling areas (J: Saint-Joseph; V: Les Versauds; E: Saint-Ennemond) in the River Morcille (four replicates per sampling area).

Fig.1

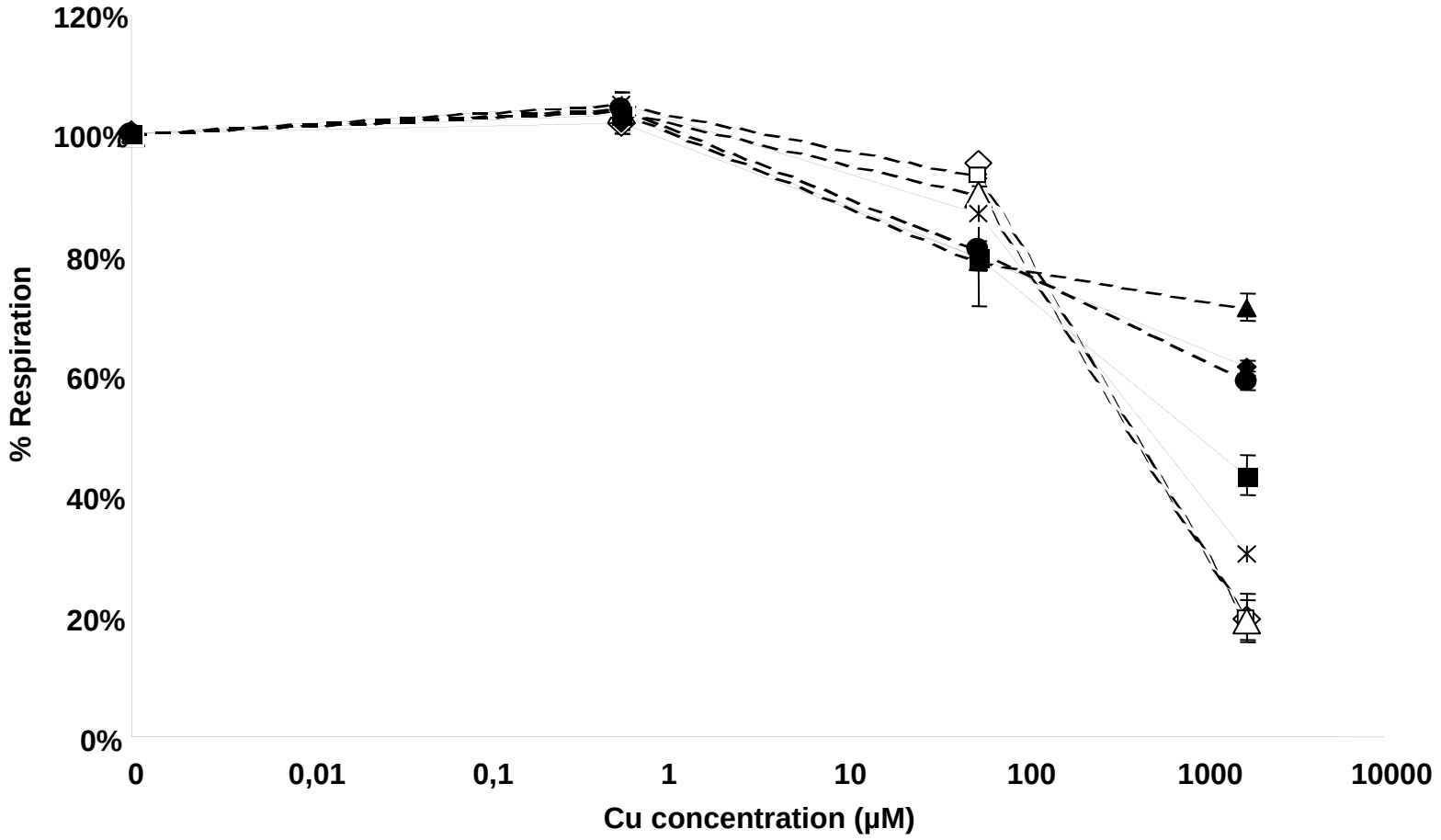

Fig.2

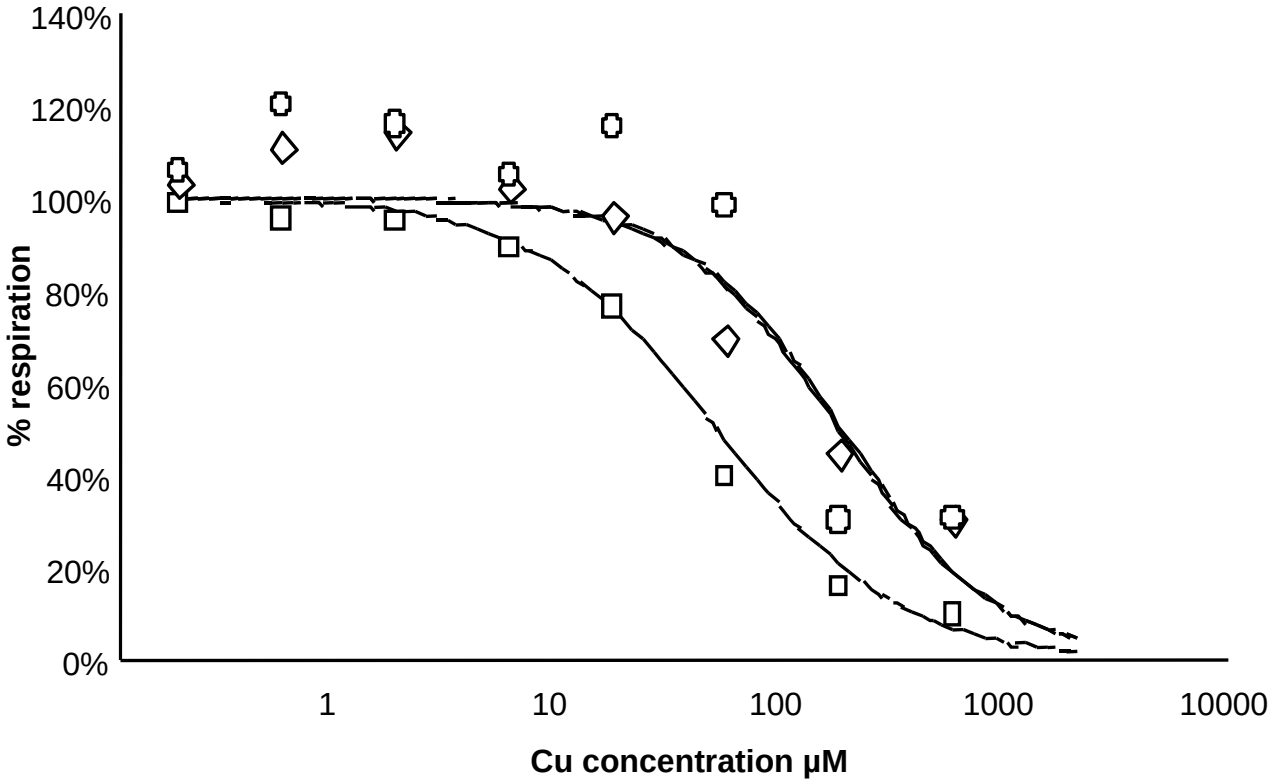

Fig.3

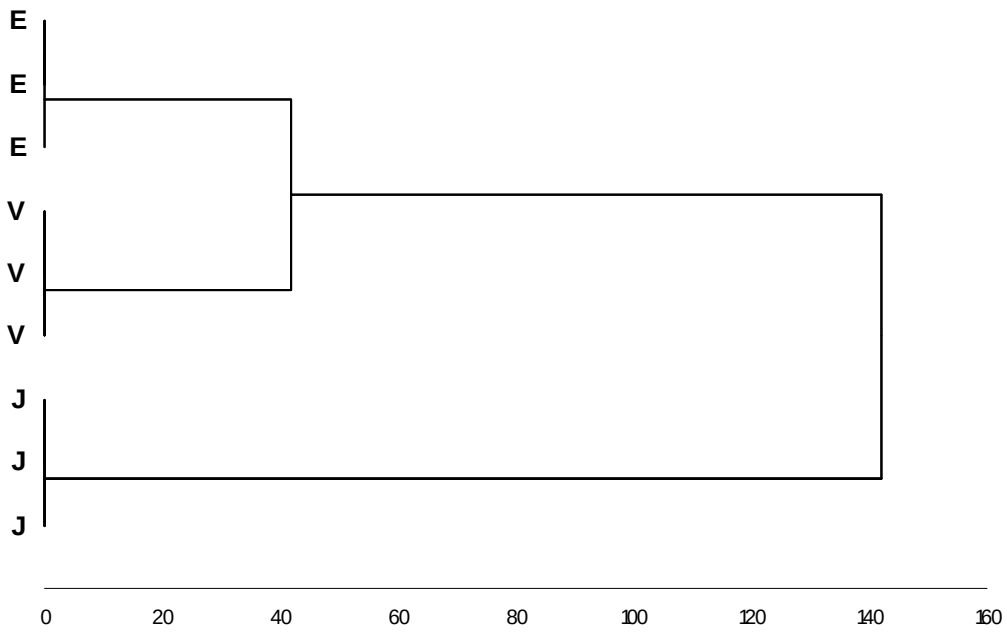

Fig.4