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## 1 Use of the MicroResp<sup>TM</sup> method to assess pollution-induced community 2 tolerance to metals for lotic biofilms

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## 21 **Abstract**

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

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

34  
35 Capsule: A modified MicroResp<sup>TM</sup> technique as a tool for measuring induced tolerance to  
36 heavy metals of microbial biofilm community.

37  
38  
39 Keywords: river biofilm; pollution-induced community tolerance; substrate induced  
40 respiration; heavy metals; MicroResp<sup>TM</sup>.

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**49 I. Introduction**

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

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

75 level physiological profile (CLPP), and more recently to assess tolerance to metals of the  
76 bacterial communities in soil and aquatic biofilms (Boivin et al., 2006; Stefanowicz et al.,  
77 2009). However, this microplate technique, based on the analysis of a bacterial community's  
78 use of several carbon sources, has major drawbacks: incomplete assessment of the whole  
79 microbial community, a long incubation time inducing bacterial selection and growth  
80 (Preston-Mafham et al., 2002) and the use of artificial culture media with buffer that may  
81 have artificial significant effects on the speciation and in consequence on the bioavailability  
82 and the toxicity of toxicants (Barranguet et al. 2003). A respirometric technique based on the  
83 analysis of the substrate-induced respiration (SIR) response (using gas chromatography  
84 analysis) was also investigated with a multiple carbon-source substrate for CLPP (Garland  
85 and Mills, 1991), and in a few studies, to test microbial tolerance to heavy metals at the  
86 community level in soils (Witter et al., 2000; Rajapaksha et al., 2004). More recently this  
87 approach was used to assess induced tolerance to copper of bacterial communities in river  
88 biofilms (Tlili et al., 2010). However, the method is still tedious, especially when assaying  
89 many microbial samples simultaneously in a multitude of bottles that have to be processed  
90 separately to measure the amount of released CO<sub>2</sub> (Chapman et al., 2007). Miniaturized  
91 systems offer the considerable advantage of allowing sample and replicate multiplication. In  
92 soil studies, Campbell et al. (2003) developed the MicroResp<sup>TM</sup> technique, which is an  
93 alternative method that combines the advantages of the Biolog<sup>TM</sup> technique, using the  
94 microplate system, and of the SIR approach with ability to measure CO<sub>2</sub> production during  
95 short-term incubation from a whole soil microbial community.

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

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

## 103 **II. Materials and methods**

### 104 **1. In-situ biofilm**

105 The biofilm used in our study was taken from the River Morcille, located in the Beaujolais  
106 vineyard area. The Morcille is a small first-order stream (7 km long) under strong agricultural  
107 pressure, essentially from vineyards, which occupy about 80% of its 8.5 km<sup>2</sup> catchment area.  
108 The Morcille watershed is part of the Long-Term Ecological Research Rhône Basin (ZABR).  
109 Regular physico-chemical surveys (hydrology, nutrients, pesticides and metals) and biological  
110 monitoring have been conducted on this site (Dorigo et al., 2007; Rabiet et al., 2008; Morin et  
111 al., 2010; Montuelle et al., 2010). Three sampling sites were selected along a gradient of  
112 increasing percentage contribution of vineyards to the catchment area: Saint-Joseph (vineyard  
113 percentage cover 11%), Les Versauds (53%) and Saint-Ennemond (65%) (see Table. 1 for  
114 physico-chemical details). Several annual surveys were performed to characterize metal  
115 contamination. In 2009 ( $n = 43$  samples), Saint-Joseph was the least polluted site with a mean  
116  $0.51 \pm 0.02 \mu\text{g.L}^{-1}$  of Cu,  $1.13 \pm 0.21 \mu\text{g.L}^{-1}$  of Zn and  $1.94 \pm 0.46 \mu\text{g.L}^{-1}$  of As. By contrast,  
117 the two other sites studied were considered polluted: Les Versauds was characterized by a  
118 mean  $2.05 \pm 0.25 \mu\text{g.L}^{-1}$  of Cu,  $0.83 \pm 0.42 \mu\text{g.L}^{-1}$  of Zn and  $11.5 \pm 0.46 \mu\text{g.L}^{-1}$  of As, and  
119 Saint-Ennemond by a mean  $3.93 \pm 0.77 \mu\text{g.L}^{-1}$  of Cu,  $7.05 \pm 3.56 \mu\text{g.L}^{-1}$  of Zn and  $9.26$   
120  $\pm 4.99 \mu\text{g.L}^{-1}$  of As. Other metals including Cd, Ni, Fe or Al were also detected at low levels  
121 (means near limits of quantification) and there was no gradient of contamination from  
122 upstream to downstream.

### 123 **Table.1**

### 124 **2. Biofilm microcosm**

125 During the different experiments with the MicroResp<sup>TM</sup>, a biofilm was grown on artificial  
126 substrates (microscope slides previously cleaned in a dilute nitric acid bath and rinsed with  
127 milli-Q water before use) in laboratory microcosms under controlled conditions. Fifteen-litter  
128 aquariums were filled with river water from the reference site (Saint-Joseph), which had been  
129 filtered through a 50 µm mesh to remove most of the grazers (water was renewed once a  
130 week). The water was kept at a temperature of  $18 \pm 2$  °C, and exposed to a light intensity of  
131  $260 \mu\text{mol m}^{-2}\text{s}^{-1}$ , with a light/dark regime of 18:6 h. Three-week-old biofilms were used for  
132 the MicroResp<sup>TM</sup> assays (Tlili et al., 2008).

### 133 **3. The MicroResp<sup>TM</sup> procedure**

134 MicroResp<sup>TM</sup> system has been described in detail by Campbell et al. (2003), but only a few  
135 ecotoxicological applications of the MicroResp<sup>TM</sup> technique have been investigated on soils  
136 (Kaufmann et al. 2006) and none on aquatic microbial communities. Here we present an  
137 adaptation of the procedure of Campbell et al. (2003) to aquatic biofilm suspensions and  
138 toxicological bioassays. MicroResp<sup>TM</sup> is a colorimetric method based on the colour change of  
139 a pH indicator dye caused by the release of CO<sub>2</sub> by heterotrophic communities. The system  
140 consists of two microplates (96 wells) placed face to face. One of these is a deep-well  
141 microplate (1.2 mL capacity, 96-deep-well microplate, NUNC) in which each well contains  
142 the microbiological sample (we used a 500 µL biofilm suspension obtained by scraping  
143 biofilm off glass substrata using a polypropylene spatula and suspending it in 0.2 µm of  
144 Nuclepore-filtered water from the reference site) with the carbon source (30 µL per well, 6.2  
145 mg of C per well). To this deep-well microplate we added the toxicant (50 µL per well). The  
146 second microplate contained the detection gel (cresol red dye (12.5 ppm), potassium chloride  
147 (150 mM) and sodium bicarbonate (2.5 mM) set in a 1% gel of noble agar (final volume 150  
148 µL per well)). The two microplates were sealed together with a silicone seal, with  
149 interconnecting holes between the corresponding wells. The assembly was clamped together  
150 and the system was incubated in the dark (to avoid any photosynthesis interference with CO<sub>2</sub>

151 release) at room temperature. CO<sub>2</sub>-trap absorbance was measured at 590 nm (Biotek Synergy  
152 HT spectrophotometer) immediately before sealing to the deep-well plate (coefficient of  
153 variation below 5%), and after incubation. Quantities of CO<sub>2</sub> produced by the microbial  
154 samples were calculated using a calibration curve of absorbance values versus CO<sub>2</sub> quantity  
155 measured by gas chromatography (MTI 200 microcatharometer). Results were then expressed  
156 as  $\mu\text{g CO}_2.\text{mg}^{-1}$  of sample.h<sup>-1</sup>. In our biofilm study, the sample was considered as organic  
157 matter (ash-free dry weight, AFDW) of the biofilm suspension tested, and measured using the  
158 protocol described by Tlili et al. (2008). Briefly, three aliquots of each homogenized biofilm  
159 suspension (2 mL) were filtered through individual 25 mm CF/C Whatman glass fibre filters  
160 (1.2  $\mu\text{m}$  pore size). Total dry matter was measured by weighing the filters after drying at  
161 105°C for 24 h. The filters were then combusted in an oven at 480°C (Nabertherm P320) for  
162 1 h, and reweighed to calculate mineral matter. AFDW was calculated by subtracting mineral  
163 matter from total dry matter.

#### 164 **4. Development of the MicroResp<sup>TM</sup> protocol**

##### 165 **Table.2**

##### 166 4.1. Incubation time

167 Campbell et al. (2003) propose an incubation time of 6 h with soil samples. However, in our  
168 case, we have to take into account the rate of CO<sub>2</sub> diffusion at the air-water interface, which  
169 may be low. To ensure a relevant measure in the well airspace of the CO<sub>2</sub> released from the  
170 sample (the biofilm), we tested a range of incubation times: 4, 6, 8, and 15 h. Individual  
171 MicroResp<sup>TM</sup> microplates were used for each incubation time. Eight deep wells of each  
172 microplate were filled with 500  $\mu\text{L}$  of biofilm suspension with 50  $\mu\text{L}$  of milli-Q water and 30  
173  $\mu\text{L}$  of D-glucose (Prolabo) (the remaining wells of the microplate were filled with 580  $\mu\text{L}$  of  
174 milli-Q water). Quantities of released CO<sub>2</sub> were measured at each incubation period, as  
175 described in Section 3.

##### 176 4.2. Pre-incubation test with toxicant

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

#### 181 4.3. Short-term bioassays

182 To validate the utility of the MicroResp<sup>TM</sup> method for short-term bioassays, using biofilms, a  
183 wide range of heavy metals was tested: CuSO<sub>4</sub> (Merck KgaA; purity≥99.0%), ZnSO<sub>4</sub>  
184 (Sigma-Aldrich GmbH), CdN<sub>2</sub>O<sub>6</sub>.7H<sub>2</sub>O (Fluka Sigma-Aldrich; purity≥99.0%), AgNO<sub>3</sub>  
185 (CARLO ERBA reagenti; purity≥99.8%), N<sub>2</sub>NiO<sub>6</sub>.6H<sub>2</sub>O (Fluka Sigma-Aldrich Chemie  
186 GmbH; purity≥97.0%), FeSO<sub>4</sub>.7H<sub>2</sub>O (Sigma Chemical co.; purity≥99.0%), CoN<sub>2</sub>O<sub>6</sub>.6H<sub>2</sub>O  
187 (Fluka Sigma-Aldrich Chemie GmbH; purity≥98.0%), AlN<sub>3</sub>O<sub>9</sub>.9H<sub>2</sub>O (Merck KgaA;  
188 purity≥98.5%) and Na<sub>2</sub>HAsO<sub>4</sub>.7H<sub>2</sub>O (Sigma-Aldrich GmbH; purity≥98.0%). Briefly, stock  
189 solutions containing 200 mM of each metal were prepared in water and stored at 4 °C, before  
190 dilution in the test wells. A semi-logarithmic series of concentrations was freshly prepared by  
191 serial dilution of the stock solutions in 0.2 µm-filtered river water. Final nominal test  
192 concentrations in the deep wells ranged from 0.5 µM to 17.2 mM (4 blanks and 4 replicates  
193 for each of the 9 concentrations). The carbon substrate used for all the SIR toxicological  
194 bioassays was D-glucose. Quantities of released CO<sub>2</sub> were measured for each bioassay as  
195 described in Section 3. Dose-response curves were plotted using CO<sub>2</sub> values produced by  
196 biofilm at each metal concentration as a percentage of CO<sub>2</sub> produced by a control biofilm  
197 (without toxicant).

### 198 **5. Application of the microrespirometric method to a field situation**

#### 199 5.1. PICT approach (short-term bioassays)

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

203 method. Briefly, biofilms were scraped from stones (500 cm<sup>2</sup> per sample) collected from the  
204 three sampling sites described previously (St. Joseph (J), Les Versauds (V) and Saint-  
205 Ennemond (E)), and homogenized in 150 mL of 0.22 µm-filtered water from the reference  
206 site (J). Biofilm suspensions were placed directly in the deep well (500 µL) and 50 µL of each  
207 tested metal (Cu, Cd or Ag) was added separately. After pre-incubation 30 µL of glucose was  
208 added to each of the deep wells, and the MicroResp<sup>TM</sup> system was assembled as described  
209 previously and incubated in the dark at room temperature (23 ± 1 °C). After incubation, the  
210 absorbance of the detection microplate was measured at 590 nm (Biotek Synergy HT  
211 spectrophotometer), the amounts of released CO<sub>2</sub> were calculated and the results were  
212 expressed in µg CO<sub>2</sub>.mg<sup>-1</sup>AFDW.h<sup>-1</sup>. Dose-response curves were plotted using CO<sub>2</sub> values  
213 produced by the biofilm at each metal concentration as a percentage of the CO<sub>2</sub> produced by  
214 control biofilms (without toxicant). The EC<sub>50</sub> values were calculated for each metal and for  
215 each site.

## 216 5.2. Catabolic diversity fingerprint

217 With a view to establishing a CLPP of the heterotrophic biofilm compartment, a multiple  
218 carbon-source substrate-induced respiration approach (using the MicroResp<sup>TM</sup> technique) was  
219 used. The different carbon substrates used were: D-glucose (Prolabo), D-fructose (Sigma  
220 chemical Co.), D-sucrose (Fluka biochemica), D-ribose (Prolabo), D-galactose (Prolabo), D-  
221 maltose (Prolabo), L-arginine (Sigma Chimie Fluka), glycine (Sigma Aldrich Chemie), L-  
222 lysine hydrochloride (Sigma Chimie, Fluka), L-glutamic acid (Sigma Aldrich Chemie) and  
223 citric acid anhydre (Sigma Aldrich Chemie). Stock solutions containing 120 mg.mL<sup>-1</sup> of each  
224 substrate were prepared in 0.22 µm-filtered Milli Q water and stored at 4 °C, until used. The  
225 pH of each solution was adjusted to river pH (7) to prevent any substrate-pH effects on  
226 microbial communities and minimize chemical artefacts due to carbonate-derived CO<sub>2</sub>. Final  
227 concentration was 6.2 mg of C per mL in each deep well. The results were expressed in µg

228 CO<sub>2</sub>.mg<sup>-1</sup>AFDW.h<sup>-1</sup>. In addition to the SIR measurements, the basal respiration (without  
229 substrate addition) was measured for each biofilm.

## 230 **6. Biofilm DNA extraction, amplification and automated ribosomal intergenic spacer** 231 **analysis (ARISA)**

232 To characterize the diversity of the river biofilm bacterial communities, we used the ARISA  
233 molecular fingerprint technique. Three replicates of 2 mL of each biofilm suspension were  
234 centrifuged at 14,000g for 30 minutes, and the supernatant was discarded and pellet stored at  
235 -80 °C before extraction. Nucleic acid extraction was performed on biofilm pellets using the  
236 FAST DNA kit (QBIogene, Illkirch, France) according to the manufacturer's instructions.  
237 The PCR conditions and the PCR template preparation for the ARISA conditions were those  
238 described by Ranjard et al. (2003). The primers used were S-D-Bact-1522-b-S-20 (3'end of  
239 16S genes) and L-D-Bact-132-a-A-18 (5'end of 23S genes) (Ranjard et al., 2003).

## 240 **7. Data treatment**

241 EC<sub>50</sub> values were calculated using the Regtox model (E. Vindimian,  
242 <http://eric.vindimian.9online.fr/>). The CLPP data sets (normalized by the basal respiration  
243 values) were subjected to principal component analysis (PCA) (XLSTAT Software Package,  
244 2009 version). The significant level was set to 5% for all the statistical tests with four  
245 replicates for each parameter. Bacterial ARISA profiles were compared with regards to the  
246 presence or absence of bands, using the Pearson similarity index. Matrices were used to  
247 perform Ward's method of hierarchical cluster analysis (HCA) using the XLSTAT Software  
248 Package (2009 version).

## 249 **III. Results and discussion**

### 250 **1. MicroResp<sup>TM</sup> protocol applied to ecotoxicological bioassays on microbial biofilms**

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

253 matter that may be different between sampling stations. Glucose mineralization activity was  
254 detected by our MicroResp<sup>TM</sup> modified design within 8 h incubation time, and net CO<sub>2</sub>  
255 production was approximately linear between 8 h and 15 h incubation time, with respiration  
256 rates of  $160.0 \pm 14.9$  and  $160.6 \pm 12.1 \mu\text{g CO}_2.\text{mg}^{-1}\text{AFDW}.\text{h}^{-1}$  for 8 h and 15 h incubation  
257 time respectively. In addition, analysis of the bacterial community profiles (ARISA) of each  
258 of the four incubation times tested showed no change in the diversity of this community (data  
259 not shown). Incubation for 8 h would have sufficed, but for practical reasons we opted to  
260 conduct our bioassays with an incubation time of 15 h (overnight).

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

274 **Fig.1.**

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

279 to each well and the detection microplate was positioned. The system was sealed and  
280 incubated for 15 h in the dark (without photosynthetic activity, which could induce an  
281 increase of the pH and interfere with the CO<sub>2</sub> release) and at room temperature (23 ± 1 °C).  
282 CO<sub>2</sub>-trap absorbance was measured at 590 nm (or better at 570 nm, Rowell, 1995)  
283 immediately before sealing to the deep well plate, and after 15 h incubation.

284 The MicroResp<sup>TM</sup> technique enabled us to plot dose-response curves with all the tested  
285 metals. The EC<sub>50</sub> obtained with the different tested metals in short-term bioassays are shown  
286 in Table.3. Compared with the other metals, silver seems to be the most toxic metal to the SIR  
287 (EC<sub>50</sub> = 3.96 μM). Aluminium (EC<sub>50</sub> = 2317.86 μM) and cobalt (EC<sub>50</sub> = 6170.63 μM) were  
288 the least toxic, while Zn, Cu and Cd had similar intermediate toxicities. However, in our study  
289 some of the metal salts used included a nitrogen source (e.g. AgNO<sub>3</sub>, N<sub>2</sub>NiO<sub>6</sub>.6H<sub>2</sub>O or  
290 CoN<sub>2</sub>O<sub>6</sub>.6H<sub>2</sub>O) that could interfere with the respiration measurements and so bias the EC<sub>50</sub>  
291 values. A second control using NaNO<sub>3</sub> in addition to glucose might be a good option to  
292 overcome this interference. Witter et al., (2000) obtained similar results when testing soil  
293 bacteria tolerance (based on SIR) to these three metals (e.g. EC<sub>50</sub> Zn value of about 250 ppm).  
294 EC<sub>50</sub> values calculated for the percentage of 95 substrates used completely within 8 days of  
295 incubation in Cu (0–300 mM)-amended BIOLOG GN plates inoculated with a river biofilm  
296 bacteria consortium were in the range 10–30 μM (Barranguet et al. 2003). Sensitivities  
297 obtained with other bioassays such as the well-known Microtox® (based on bioluminescence  
298 reduction of the marine bacterium *Vibrio fischeri*) are also comparable to our range of EC<sub>50</sub>  
299 values (e.g. EC<sub>50</sub>(5 mn) = 941 μM CdCl<sub>2</sub> and 168 μM CuCl<sub>2</sub> and EC<sub>50</sub>(15mn) = 421 μM  
300 CdCl<sub>2</sub> and 49 μM CuCl<sub>2</sub>, in Macken et al. 2009). Therefore, our dose-response curves and  
301 EC<sub>50</sub> obtained with the SIR, related to heterotrophic organisms, confirm that the MicroResp<sup>TM</sup>  
302 technique could be a useful method to classify heavy metals according to their toxicity toward  
303 river biofilms.

304 **Table.4.**

## 2. Application of the microrespirometry method to a field situation

### 2.1. Copper-induced tolerance

Cu EC<sub>50</sub> values were estimated to evaluate tolerance to this pollutant of the biofilm communities sampled at three sites along the River Morcille. EC<sub>50</sub> 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<sub>50</sub> 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 bioavailability 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

336 are controlled and standardized, and that different physicochemical parameters are  
337 homogeneous and similar during short-term exposure of the tested communities. Blanck  
338 (2002) stipulated that bioavailability may interfere in the PICT detection as a confounding  
339 factor, for example when the waters used in the short-term tests are different. This could then  
340 affect the  $EC_x$  values obtained and finally the PICT signal may represent a bioavailability  
341 gradient. However, this remark concerns a regional survey (Blanck et al., 2003) that compared  
342 very different sampling sites (from different rivers and countries) in the context of bioassays  
343 that used the filtered natural water from each sampling area. This was not the case in our  
344 study, where we used the same filtered water of the upstream sampling area **J** (pH values in  
345 the range 7.2–8) for periphytic suspensions and toxic solutions of all bioassays and in the  
346 context of a small river basin. In addition, in a previous study we measured pH in the biofilm-  
347 river suspension (using water from site **J**), glucose and copper added at the beginning and end  
348 of the short-term incubation, and observed no pH changes during the bioassays despite  
349 demonstrating an induced tolerance to copper based on the substrate-induced respiration as a  
350 functional parameter (measured by gas chromatography, data not shown). Rusk et al. (2004)  
351 investigated tolerance of soil biological nitrification to metals using sterilized, metal-treated  
352 soils from the reference area reinoculated with a similar soil containing the microbial  
353 communities to be tested. Our approach using filtered water from a reference area (upstream)  
354 to perform the PICT bioassays, in which we suspended periphyton scraped from the different  
355 investigated sites, is comparable to the approach of Rusk et al. (2004), who wanted to exclude  
356 the potentially confounding effects of variations in metal bioavailability in their tolerance  
357 bioassays. We can conclude that the bioassays conducted during our study were performed  
358 under homogeneous standardized conditions and minimized variations in metal bioavailability  
359 among the microbial samples tested.

360 **Table.4. Fig.2. Fig.3.**

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

373 This first biofilm ecotoxicological bioassays using MicroResp<sup>TM</sup> applied in a river  
374 contamination assessment context, highlights the efficiency of this technique for measuring  
375 the induced tolerance to metals of the heterotrophic biofilm community based on SIR.

## 376 2.2. Co-tolerance assessment

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

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

### 398 2.3. Community-level physiological profiles

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

413 Fig.4.

414 **VI. Conclusion and perspectives**

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

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## Postprint

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Tlili, A., Maréchal, M., Montuelle, B., Volat, B., Dorigo, U., Berard, A. (2011). Use of the MicroResp (TM) method to assess pollution-induced community tolerance to metals for lotic biofilms. Environmental Pollution, 159 (1), 18-24. DOI : 10.1016/j.envpol.2010.09.033

587 Table.1. Means ( $\pm$  standard deviation,  $n = 43$ ) of selected chemical parameters (pH; cond.:  
 588 conductivity [ $\mu\text{S.cm}^{-1}$ ]; DOC [ $\text{mg.L}^{-1}$ ];  $\text{NH}_4^+$  [ $\text{mg.L}^{-1}$ ];  $\text{NO}_2^-$  [ $\text{mg.L}^{-1}$ ];  $\text{NO}_3^-$  [ $\text{mg.L}^{-1}$ ] and  
 589  $\text{PO}_4^{3-}$  [ $\text{mg.L}^{-1}$ ]) obtained from the three sampling areas (J: Saint-Joseph; V: Les Versauds and  
 590 E: Saint-Ennemond) over two years (2009 and 2010).

Site	pH	cond	DOC	$\text{NH}_4^+$	$\text{NO}_2^-$	$\text{NO}_3^-$	$\text{PO}_4^{3-}$
<b>J</b>	7.27 $\pm 0.29$	161.72 $\pm 17.65$	2.67 $\pm 0.88$	0.04 $\pm 0.03$	0.02 $\pm 0.01$	6.82 $\pm 1.28$	0.07 $\pm 0.03$
<b>V</b>	7.41 $\pm 0.27$	201.33 $\pm 18.57$	3.28 $\pm 0.91$	0.05 $\pm 0.04$	0.03 $\pm 0.01$	7.58 $\pm 3.03$	0.23 $\pm 0.13$
<b>E</b>	7.51 $\pm 0.24$	233.61 $\pm 33.52$	4.52 $\pm 1.82$	0.08 $\pm 0.04$	0.06 $\pm 0.06$	7.33 $\pm 2.69$	0.28 $\pm 0.17$

610 Table. 2. Chronological summary of the different experiments and steps performed during the  
 611 study  
 612

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

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615 Table. 3. Sensitivity (expressed as EC<sub>50</sub> in μM) of the heterotrophic biofilm community to  
 616 different tested metals in short-term bioassays using MicroResp<sup>TM</sup>. C.I. confidence interval  
 617 (*n* = 4; *α* = 0.5)

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

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646 Table. 4. EC<sub>50</sub> values (in μM of copper, silver or cadmium) for the three sampling sites (J:  
 647 Saint-Joseph; V: Les Versauds; E: Saint-Ennemond). C.I. confidence interval ( $n = 4$ ;  $\alpha = 0.5$ ).  
 648

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

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666 Fig. 1. Respiration values (expressed as a percentage of control) after short-term bioassay  
667 with different pre-incubation periods (▲ 0; ◆ 0.25; ● 0.5; ■ 1; \* 2; ◇ 3 ; □ 4 and △ 6 h)  
668 before addition of the carbon substrate (glucose). Error bars represent standard deviations ( $n =$   
669 4).

670  
671 Fig. 2. Copper tolerance of heterotrophic communities at three sampling areas along a copper  
672 contamination gradient in the River Morcille. Symbols for each area (◆: Saint-Joseph; ■: Les  
673 Versauds; ▲: Saint-Ennemond), represent average SIR expressed as a percentage of the  
674 corresponding controls and obtained during the MicroResp<sup>TM</sup> short-term bioassay. Dose-  
675 response curves (◇: Saint-Joseph; □: Les Versauds; △: Saint-Ennemond) represent the Hill  
676 equation model applied to the same SIR measurements. Parameters were calculated by  
677 nonlinear regression and statistics (four replicates) were obtained using a Bootstrap-Monte-  
678 Carlo simulation (from Regtox model).

679  
680 Fig 3. Cluster analysis (hierarchical ascendant classification based on Pearson correlation  
681 coefficient) of the bacterial community (ARISA analysis of PCR amplified IGS gene  
682 fragments) from three sampling areas (J: Saint-Joseph; V: Les Versauds; E: Saint-Ennemond)  
683 on the River Morcille (three replicates per sampling area).

684  
685 Fig 4. Principal component analysis (PCA) of community-level physiological profiles (CLPP)  
686 of biofilms measured with the MicroResp<sup>TM</sup> method and normalized to basal respiration, from  
687 three sampling areas (J: Saint-Joseph; V: Les Versauds; E: Saint-Ennemond) in the River  
688 Morcille (four replicates per sampling area).

Fig.1

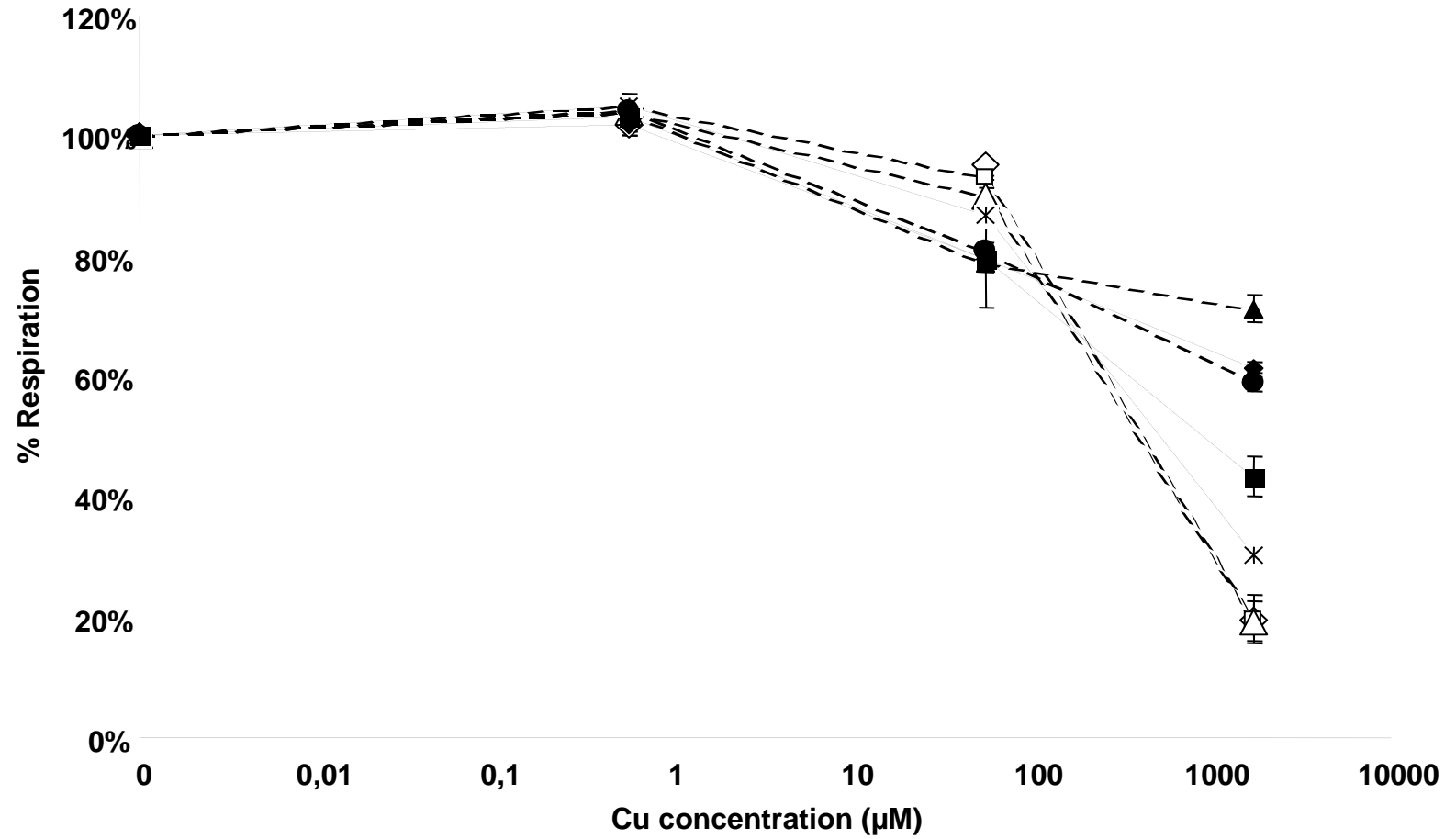


Fig.2

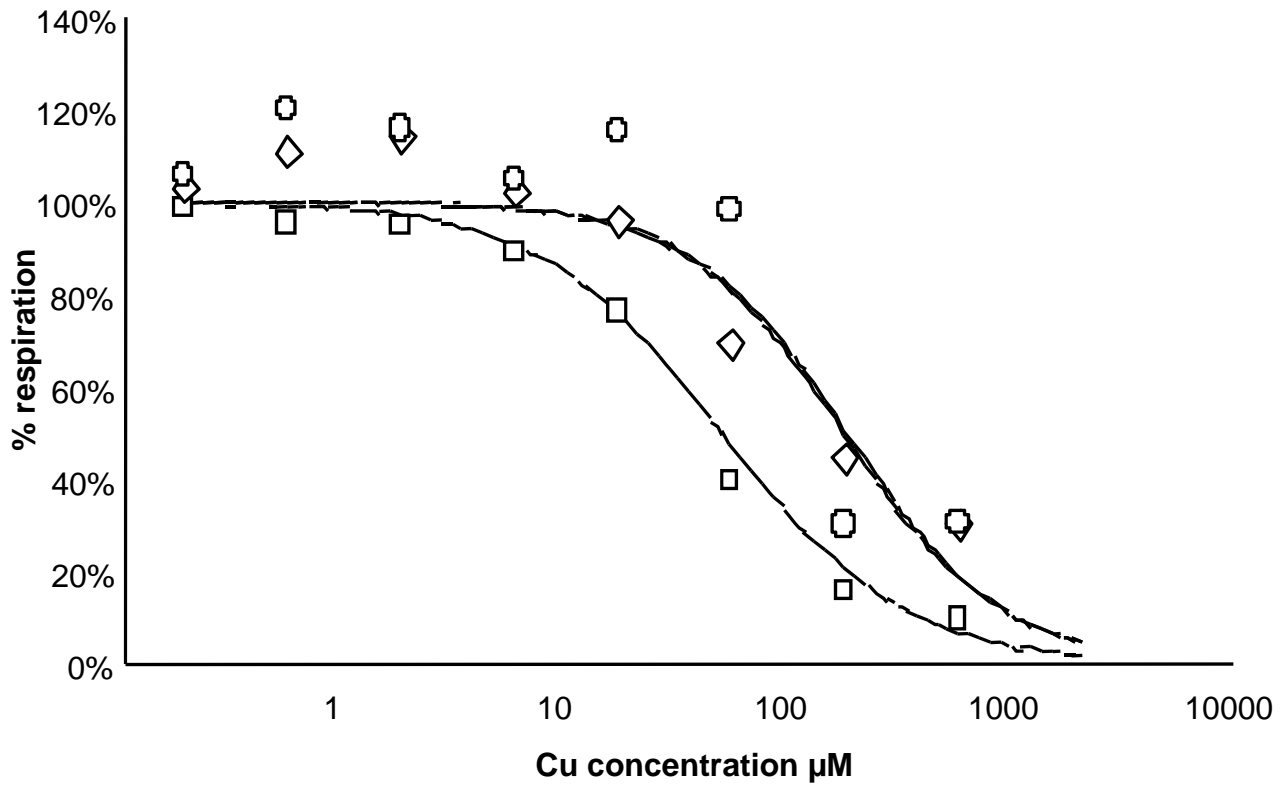


Fig.3

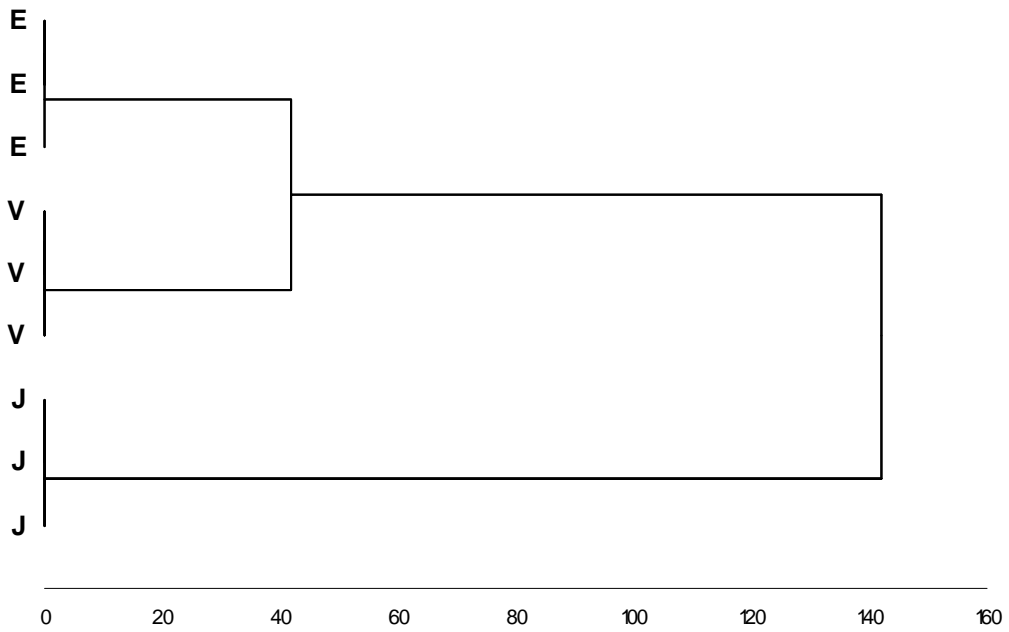


Fig.4

