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1 **Comparative *in vitro/in situ* approaches to three biomarker responses of**
2 ***Myriophyllum alterniflorum* exposed to metal stress**

3
4 Raphaël DECOU^{a*}, Servane BIGOT^a, Philippe HOURDIN^a, David DELMAIL^{ab} and Pascal
5 LABROUSSE^a

6 ^a University of Limoges, PEIRENE, EA 7500, F-87000 Limoges, France

7 ^b University of Rennes 1, UMR 6118 Géosciences, F-35043 Rennes, France

8
9 **ABSTRACT**

10 Surface water pollution by trace metal elements constitutes problems for both public and
11 terrestrial/aquatic ecosystem health. *Myriophyllum alterniflorum* (alternate watermilfoil), an
12 aquatic macrophyte known for bioaccumulating this type of pollutant, is an attractive species
13 for plant biomonitoring within the scope of environmental research. The two metal elements
14 copper (Cu) and cadmium (Cd) are considered in the present study. Cu is essential for plant
15 development at low concentrations, while very high Cu concentrations are detrimental or even
16 lethal to most plants. On the other hand, Cd is usually toxic even at low concentrations since it
17 adversely affects the physiological plant functions. In order to check whether watermilfoil could
18 be used for the *in situ* biomonitoring of Cu or Cd pollution in rivers, the plant biomarker
19 sensitivity is first tested during long-term *in vitro* assays. Three markers specific to oxidative
20 stress (glucose-6-phosphate dehydrogenase, malondialdehyde and α -tocopherol) are evaluated
21 by varying the pollutant concentration levels. Given the absence of effective correlations
22 between Cu and all biomarkers, the response profiles actually reveal a dependency between Cd
23 concentration and malondialdehyde or α -tocopherol biomarkers. Conversely, preliminary *in*
24 *situ* assays performed at 14 different localities demonstrate some clear correlations between all
25 biomarkers and Cu, whereas the scarcity of Cd-contaminated rivers prevents using the statistical

26 data. Consequently, the three indicated biomarkers appear to be effective for purposes of metal
27 exposure analyses; moreover, the *in situ* approach, although preliminary, proves to be
28 paramount in developing water biomonitoring bases.

29

30 **KEYWORDS:** Aquatic plant, Biomarkers, Trace metal elements, Oxidative stress, Statistical
31 correlation.

32

33 *Corresponding author: raphaeldecou@yahoo.fr

34 Laboratoire de Botanique et Cryptogamie - PEREINE EA7500

35 Faculté de Pharmacie, 2, rue du Dr Marcland, 87025 Limoges Cedex, France

36 Phone: +33 (0)5 55 435 841

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38

39 INTRODUCTION

40 Biomonitoring refers to the use of biomaterials or organisms in analyzing certain characteristics
41 of the biosphere. Water metal and metalloid pollution constitutes one of these assessable
42 characteristics, especially in surface waters like lakes, rivers, estuaries, coastal zones, seas and
43 oceans. Metal pollutants, also called trace metal elements (or TME), result from not only natural
44 geochemical processes but also demographic growth and human activities, such as mining,
45 smelting, warfare, military training, the electronics industry, fossil fuel consumption, waste
46 disposal, agrochemical use and irrigation (Liu *et al.*, 2018). Nonetheless, mining as driven by
47 human demand for minerals and metals is actually the primary cause of the environmental
48 release of TME, which in turn increase pressure on the ecosystems (Gu, 2018).

49 Contrary to most organic pollutants, TME do not degrade in surface waters, sediments or soil
50 whenever long removal processes are involved, thus resulting in long-term impacts on

51 ecological communities (Hayes *et al.*, 2018). Depending on the TME under consideration, the
52 effects on organisms differ according to the concentration, chemical structure, bioavailability
53 and utility of the particular TME in biological processes. For example, at low concentrations,
54 copper (Cu) is an essential compound involved in many metabolic pathways, while cadmium
55 (Cd) is considered to be non-essential and toxic for the biological development of organisms
56 by virtue of inhibiting various processes in plant metabolism (Delmail *et al.*, 2011b, 2011c).
57 Low Cd concentrations however may be beneficial, as observed for example on *Miscanthus*
58 *sinensis* (Arduini *et al.*, 2004) and *Triticum aestivum* growths (Lin *et al.*, 2007). In any case, all
59 TME, whether essential or not, tend to be toxic at high concentrations and accumulate in
60 organisms.

61 The increase of TME concentrations in ecosystems over the past few decades, as observed in
62 the food chain (Zhang *et al.*, 2017), has led to establishing new regulations to better protect
63 both the environment and biodiversity. Along these lines, the European Water Framework
64 Directive (2000/60/EC) was instituted in 2000 to maintain or restore the ecological and
65 chemical qualities of surface waters and groundwater. Accordingly, such objectives require
66 constantly evaluating water quality by means of biomonitoring. Fish, mollusks and
67 invertebrates are extensively used as bioindicators of metal pollution (Van Praet *et al.*, 2014;
68 Wang and Lu, 2017; Bouzahouane *et al.*, 2018; Souza *et al.*, 2018), although aquatic plants are
69 also increasingly being investigated for these ecological surveys (Ferrat *et al.*, 2003; Krayem *et*
70 *al.*, 2016; Reale *et al.*, 2016). More specifically, the analyses of association, composition and
71 richness of plant species during *in situ* studies have become even more widespread and in depth.
72 In addition to the level of metal accumulation in organisms, the physiological, biochemical and
73 anatomical changes in organs or in the entire organism have for a number of years provided an
74 interesting way forward in the field of water biomonitoring.

75 *Myriophyllum* L. (Haloragaceae R.By.), a cosmopolitan and submerged aquatic macrophyte
76 genus comprising some 60 species (Lü *et al.*, 2017), is able to accumulate chemicals, as
77 observed in several studies, i.e. *in situ* accumulation of cobalt, lead, zinc, nickel, iron and
78 manganese in *Myriophyllum aquaticum* (Harguinteguy *et al.*, 2013, 2016) or in *Myriophyllum*
79 *spicatum* (Galal and Shehata, 2014). Cu, Cd or arsenic hyperaccumulation in *Myriophyllum*
80 *alterniflorum* has also been evaluated from *in vitro* cultures (Delmail *et al.*, 2011b, 2011c; 2013;
81 Krayem *et al.*, 2016; Krayem *et al.*, 2018). In these last studies however and in contrast with
82 other studies, biological changes were also measured through biomarker response analyses
83 focusing on scavengers indicating the oxidative stress generated by reactive oxygen species
84 (ROS) and/or antioxidant systems.

85 This study has been conducted to investigate the *in vitro* sensitivity of three *M. alterniflorum*
86 biomarkers, which are known and demonstrated to be directly or indirectly correlated with the
87 oxidative stress, to various Cd or Cu concentrations. One of these biomarkers, glucose-6-
88 phosphate dehydrogenase (G6PDH; EC 1.1.1.49), is a cytosolic or plastidic enzyme (Hauschild
89 and von Schaewen, 2003); it catalyzes the first step in the pentose-phosphate shunt by oxidizing
90 glucose-6-phosphate (G6P) to 6-phosphogluconate (6PG) as well as by transforming
91 nicotinamide adenine dinucleotide phosphate (NADP) into reduced nicotinamide adenine
92 dinucleotide phosphate (NADPH). As such, this enzyme regulates the NADPH level, which
93 itself is necessary for GSH regeneration and consequently for oxidative stress resistance. In
94 contrast, malondialdehyde (MDA), the second biomarker tested herein, is a major product of
95 the membrane lipid peroxidation by ROS, which justifies its use as a bioindicator of oxidative
96 stress. Lastly, α -tocopherol (also called vitamin E) constitutes the third investigated biomarker
97 and is directly correlated with ROS scavenging by virtue of being considered as a key lipophilic
98 radical-scavenging antioxidant *in vivo* (mainly in chloroplasts), in preventing lipid peroxidation
99 and contributing to the physical stability of membranes (Nacka *et al.*, 2001; Munné-Bosch,

100 2005). Level changes in the α -tocopherol response to free radicals make this biomarker a good
101 candidate for metal stress detection.

102 In accordance with the specificity observed for each biomarker with respect to these pollutants,
103 a preliminary and complementary *in situ* study was conducted across 14 localities selected on
104 the basis of both their TME contents and anthropogenic impact type. Consequently, this 35-day
105 assay allowed establishing correlation analyses between TME and biomarkers despite the
106 presence of plants subjected to variable environmental parameters, such as physicochemical
107 parameters, light, rainfall or pollutant level variations. Biomarker response comparisons
108 between *in vitro* and *in situ* assays will be discussed and could offer critical input in validating
109 the use of this aquatic plant as an *in situ* bioindicator.

110

111 **MATERIALS AND METHODS**

112 **Micropropagation and plant growth**

113 *Myriophyllum alterniflorum* clones were grown *in vitro* within culture boxes (Steri Vent High
114 Model 80x110x100 mm, KALYS SA, France) containing 300 mL of Murashige and Skoog
115 (MS, Kalys, France) medium (pH 5.8), supplemented by 3% sucrose (Murashige and Skoog,
116 1962). The plants were maintained in a growth cabinet set at $24^{\circ} \pm 2^{\circ}\text{C}$, with a photoperiod of
117 16 h and a light intensity of $7.47 \pm 3.15 \text{ W/m}^2$ (neon Supra'Lux Actizoo 30 W, France), as
118 described in Delmail *et al.* (2011b).

119

120 ***In vitro* assays in artificial media supplemented by copper or cadmium**

121 After 35 days, plant clones were acclimatized over 2 weeks in a new medium with a chemical
122 composition identical to the Vienne River (i.e. oligotrophic medium), as described in Delmail
123 *et al.* (2011a). 450 clones were thus transferred into culture boxes containing 300 mL of
124 oligotrophic medium (apportioned 10 explants per box). CuSO_4 , 5 H_2O or CdCl_2 , 2.5 H_2O

125 (VWR, USA) was added aseptically to the medium in 15 boxes (for each tested concentration)
126 in order to obtain a final concentration ranging either from 5 to 100 $\mu\text{g CuSO}_4/\text{L}$ (corresponding
127 to 1.3 - 25.5 $\mu\text{g Cu}/\text{L}$) or from 0.5 to 10 $\mu\text{g CdCl}_2/\text{L}$ (corresponding to 0.2 - 4.9 $\mu\text{g Cd}/\text{L}$). These
128 concentrations were chosen for their similarity to those observed in the Vienne River (0.5 - 12.6
129 $\mu\text{g Cu}/\text{L}$ and 0.04 - 1.4 $\mu\text{g Cd}/\text{L}$). The other 15 boxes served as controls. The experiment was
130 performed for 27 days, and biological measurements were recorded every other day.

131

132 ***In situ* plant deployment and sampling**

133 The micropropagated patches from the MS medium were placed for 2 weeks in new culture
134 boxes for acclimatization in a 300-mL oligotrophic medium. Next, 12 watermilfoil patches
135 were transferred into wire-mesh cages (40x30 cm) in sandy stream sediments. The plants were
136 deployed in the 14 test localities (see Figs. 1A and B) for 35 days, and some of the watermilfoil
137 clones from each location were sampled at 1, 3, 6, 8, 14, 21, 28 and 35 days after *in situ*
138 introduction. The choice of each locality corresponded to potential pollution origins close to
139 the streams, e.g. industrial zones, a junkyard, water treatment plant, urbanized area, farming
140 zone, tannery waste site, and road and expressway traffic, as shown on the map (Fig. 1C; the
141 poor anthropogenic zone used as our reference was located in locality “10”). The control (T0)
142 corresponded to acclimatized clones approx. 14 days *in vitro* that were never placed in the river.
143 Each watermilfoil sample was then weighed and stored at -80°C for further analyses.

144

145 **Protein extraction and quantification**

146 For the *M. alterniflorum* enzyme activities presented below, protein extractions were performed
147 according to a method based on the protocol developed by Srivastava *et al.* (2006). Fresh plant
148 material (200 mg) was ground at $+4^\circ\text{C}$ in a 800- μL buffer containing 50 mM Tris-HCl (pH
149 7.0), 3.75% polyvinyl pyrrolidone (PVP) (w/v), 0.1 mM ethylenediaminetetraacetic acid

150 (EDTA) and 1 mM phenylmethylsulfonyl fluoride (PMSF). All these compounds were obtained
151 from Sigma-Aldrich (USA). The extracts were centrifuged at 10,000 g for 10 min at +4°C (3-
152 18K, Sigma-Aldrich, USA), and the total protein contents were quantified using the Bradford
153 assay (Bradford, 1976). 50 µL of protein extract were added to a cuvette containing 1.5 mL of
154 Bradford Reagent (Sigma-Aldrich, USA). After a 15-min incubation at room temperature, the
155 absorbance of the protein-dye complex was read at 595 nm (Helios Beta, Thermo Spectronic,
156 USA). The protein concentrations were determined by comparison with the standard curve
157 prepared using the protein standard, i.e. bovine serum albumin (BSA, Sigma-Aldrich).

158

159 **Watermilfoil enzyme activity**

160 The G6PDH activity was assayed according to a method based on the protocol developed by
161 Shanzhi *et al.* (2004). The rate of NADPH formation, which is proportional to the G6PDH
162 activity, was measured spectrophotometrically as an increase in absorbance at 340 nm. The
163 production of a second molar equivalent of NADPH by 6-phosphogluconate dehydrogenase
164 (6PGDH) was prevented by using β-mercaptoethanol, an SH-reagent inhibitor of 6PGDH.
165 Hence, the 3-mL reaction mixture contained, in order: 0.15 M Tris-HCl (pH 8.0), 0.1 mM
166 NADP, 1 mM PMSF, 3 mM β-mercaptoethanol, and 2% protein extract (v/v). All compounds
167 were again obtained from Sigma-Aldrich (USA). The increase in absorbance was monitored
168 for 3 min with the Helios Beta Spectrophotometer (Thermo Spectronic, USA). The G6PDH
169 activity was expressed in terms of µkat/mg protein. One µkat equals the amount of protein
170 required for an increase of 1.66E-4 Abs₃₄₀/min under assay conditions.

171

172 **Estimation of lipoperoxidation**

173 Malondialdehyde (MDA) quantification is based on the method derived by Heath and Packer
174 (1968). 250 mg of three watermilfoil clones (apex) were ground at +4°C with a pestle in a

175 mortar containing 1 g of Fontainebleau sand and 12.5 mL of 1% trichloroacetic acid (m/v; TCA,
176 Sigma-Aldrich, USA). The extract was then centrifuged at 2,600 g for 10 min (3-18K, Sigma-
177 Aldrich, USA). One mL of the supernatant was added to 1 mL of 0.5% thiobarbituric acid
178 (TBA; prepared in 20% TCA, m/v, Sigma-Aldrich, USA), and the mix was boiled at +100°C
179 for 30 min in a water bath before being chilled on ice for 5 min. Next, the samples underwent
180 10 min of 2,600-g centrifugation at +4°C, and the absorbance was read at 532 nm in a
181 spectrophotometer (Helios Beta, Thermo Spectronic, USA). The MDA concentration was
182 calculated using both the molar extinction coefficient (i.e. $1.55\text{E-}3\text{ cm}^2\cdot\text{mmol}^{-1}$) and Beer-
183 Lambert Law (Beer, 1852); it was expressed in mmol/g FW.

184

185 **Vitamin E (α -tocopherol)**

186 This molecule occurs naturally in alternate watermilfoil, and its level changes reflect an
187 oxidative stress relative to ROS production. The α -tocopherol concentration was assayed as
188 described in the HPLC protocol developed specifically for *M. alterniflorum* (Delmail *et al.*,
189 2011a).

190

191 **Trace metal elements in water and plants**

192 The copper and cadmium contained in the samples were measured in triplicate using ICP-MS
193 (SCIEX-ELAN 6100 DRC, Perkin-Elmer, USA) from both *in vitro* collected *M. alterniflorum*
194 and freshly sampled waters (for each locality, water was sampled once on Days 1, 3, 6, 8, 14,
195 21, 28 and 35 after plant deployment in the rivers). For plant mineralization, watermilfoil clones
196 were carefully rinsed in running tap water, thoroughly washed with distilled water and blotted
197 dry; 500 mg of fresh weight were digested in 2.5 mL HNO₃ 69% (Fisher chemical, Thermo
198 Fisher Scientific, USA) at 60°C for 48 h. 500 microliters of the solution were diluted in 2 mL
199 of aqueous solution (4.5% n-butanol, 0.1 g/L ammonia, 0.1 g/L EDTA, 1% germanium as the

200 internal standard) and then introduced into the nebulizer. Five-point reference scales were
201 applied according to the same protocol as before (from 0.1 to 10 µg/L Cd and 0.5 to 50 µg/L
202 Cu). For each sample, five technical replicates were performed. The limits of quantification
203 (LQ) were: 0.1 µg/L for Cd, and 0.5 µg/L for Cu. The Plasma Power, Plasma Gas Flow and
204 Auxiliary Gas Flow equaled 1,350 W, 15 L/min and 1.175 L/min, respectively.

205

206 **Statistical analyses**

207 Physiological measurements were carried out in triplicate. The software R 2.11.0 was used for
208 statistical analysis purposes. The normality of the measurement data matrix was tested with the
209 Multivariate Shapiro-Francia test (Delmail *et al.*, 2011d), which indicated that the results did
210 not follow a normal distribution (i.e. $p > 0.05$). Consequently, the non-parametric Kendall
211 correlation test was able to assess the relationship between biomarkers and metal exposure. No
212 analyses of variance can be performed here as residues followed a heteroscedasticity (Kullback
213 test).

214

215

216 **RESULTS**

217 ***Myriophyllum alterniflorum* biomarker response to Cu**

218 During the initial hours of 100 µg/L CuSO₄ treatment, *M. alterniflorum* accumulated Cu very
219 quickly (as the watermilfoil Cu content increased by a factor of 21.6 during the first day, i.e.
220 from 0.76 to 16.4 ng Cu/mg Dry Weight (DW), see Fig. 2A). This accumulation was also
221 observed for lower-level CuSO₄ treatments (data not shown). Beyond this initial period,
222 between 1 and 27 days of treatment, the Cu phytoaccumulation increased slowly, with an
223 apparent stabilization around 22 ng Cu/mg DW during the last 10 days of analysis. Due to these

224 homogeneous Cu bioaccumulations from Days 1 through 27, the origins of heterogeneous
225 differences in biomarker profiles were difficult to define (Fig. 3).

226

227 The entire G6PDH profiles remained moderately homogeneous throughout the assay yet by
228 focusing on a shorter period (from 3 to 15 days), greater profile homogeneity was observed.
229 During this period, the maximum homogeneity was obtained not only for 5 and 25 $\mu\text{g CuSO}_4/\text{L}$
230 but also for 50 $\mu\text{g CuSO}_4/\text{L}$. For these particular activity profiles, the relative homogeneity can
231 be demonstrated through similar peaks: initial peak at 3 days (+168.9%, +309.9% and +167.4%,
232 respectively - Note: The percentage values expressed here correspond to the total increase
233 between peak beginning and peak end); second peak at 11 days (+367.8%), 9 days (+425.4%)
234 and 7 days (+132.1%); and third peak at 15 days (+178.4% and +302.0%) and 11 days
235 (+173.1%). In contrast, the activity profiles at 10 and 100 $\mu\text{g CuSO}_4/\text{L}$ shared more similarities
236 during the final 13 days of analysis: i.e. one peak (+508.7%) at 21 days for 10 $\mu\text{g CuSO}_4/\text{L}$ and
237 in the form of 2 peaks (+194.4% at 17 days and +183.5% at 21 days) for 100 $\mu\text{g CuSO}_4/\text{L}$;
238 another peak at 27 days (+55.0% and +332.9%) for 10 and 100 $\mu\text{g CuSO}_4/\text{L}$, respectively.

239

240 According to the MDA profiles (Fig. 3), biomarker responses increased between Days 11 and
241 21 for all CuSO_4 concentrations (except 5 $\mu\text{g CuSO}_4/\text{L}$). Although the MDA level seems to be
242 higher in response to the increase of Cu in the medium, no apparent correlation between these
243 two parameters could be detected. The maximum increase for each treatment was: +3.4% at 17
244 days, +10.1% at 13 days, +12.0% at 11 days, +11.9% at 17 days, and +10.9% at 17 days for 5,
245 10, 25, 50 and 100 $\mu\text{g CuSO}_4/\text{L}$, respectively. Beyond this period, the MDA content decrease
246 was measured as of Day 23.

247

248 *M. alterniflorum* vitamin E content in response to various CuSO₄ concentrations revealed
249 slightly similar profiles during the 27-day treatment (Fig. 3), which indicates the lack of
250 correlation between vitamin E level and Cu concentration. All profiles showed a sharp increase
251 in vitamin E during the first few days, appearing earlier for high Cu concentrations (compared
252 to the control level: +18.9% at 9 days, +7.2% and +4.9% at 5 and 9 days (two adjacent peaks),
253 +11.7% at 7 days, +7.4% at 3 days, and +3.8% at 5 days for 5, 10, 25, 50 and 100 µg CuSO₄/L,
254 respectively). Interestingly, at around 15 ± 2 days, a distinct yet rather weak decrease in vitamin
255 E could be observed for all profiles (except at 5 µg CuSO₄/L), followed by a plateau phase
256 occurring later for high Cu concentrations (at 17, 19 and 21 days for 10, 25 and 50-100 µg
257 CuSO₄/L, respectively).

258

259 ***Myriophyllum alterniflorum* biomarker response to Cd**

260 Similar to the previous comments on Cu phytoaccumulation in *M. alterniflorum*, Cd
261 accumulation presented similar profiles during the 27 days of 10 µg/L CdCl₂ treatment (Fig.
262 2B). By the day after treatment, the clones had absorbed 0.1 ng Cd/mg DW, and this value
263 reached 0.21 ng Cd/mg DW at 17 days. A plateau phase was observed next. For the other CdCl₂
264 concentrations used as treatments in the synthetic media, the Cd accumulation profiles were
265 analogous to the previous one, but with the plateau phase appearing more quickly (data not
266 shown). Once again, like for the Cu treatment presented above, this fast Cd bioaccumulation in
267 plants complicates the definition of biomarker response origins during the 1 to 27-day period
268 (Fig. 4). Nevertheless, early biomarker responses due to these quick TME accumulations could
269 be expected. It therefore seems important to focus on shorter time segments to explain the
270 homogeneity and heterogeneity zones between the five response profiles of each biomarker.

271

272 Regarding the G6PDH profiles in Figure 4, it appeared to be difficult at first to find
273 homogeneous responses between each CdCl₂ treatment even though 1 and 4 µg CdCl₂/L
274 treatments were slightly similar over the final 7 days (respectively one peak at 21 days:
275 +472.1% and +226.9%, and another peak at 27 days: +229.3% and +785.0%). According to
276 biomarker response intensities over the period from Day 0 to Day 13, an analogy can be
277 observed between 0.5 and 10 µg CdCl₂/L treatments as well (+253.1% at 5 days and +131.8%
278 at 1 day / +263.8% and +190.6% at 9 days, respectively). On the contrary, the 7 µg CdCl₂/L
279 profile reveals greater peak intensities (+1,029.6% at 5 days and +563.0% at 13 days).

280
281 The MDA profiles graphically indicate a biomarker response clearly sensitive to the Cd
282 concentration between Days 11 and 17 (Fig. 4). In addition to this observation, the biomarker
283 response is dependent on Cd concentration. The maximum MDA content actually equals: 14.73
284 ± 0.19, 15.7 ± 0.37, 16.45 ± 0.0, and 17.85 ± 0.37 mmol/g FW from 1 to 10 µg CdCl₂/L. It is
285 interesting to note that all these values are obtained at 13 days for practically every MDA
286 profile.

287
288 Like with the MDA biomarker, vitamin E content is also dependent on Cd concentration,
289 although it decreased between 9 and 19 days with a minimum reached at 13 days for all CdCl₂
290 concentrations except 0.5 µg/L (Fig. 4). At this latter concentration, the vitamin E content
291 profile in *M. alterniflorum* completely differs from the others. This result suggests that the low
292 Cd supplementation in the oligotrophic medium did not significantly influence the biomarker
293 response.

294

295

296 ***In situ Myriophyllum alterniflorum* biomarker responses**

297 As a complement to the previous *in vitro* results, a broad experiment based on 14 different
298 locations was performed on *M. alterniflorum*; this assay lasted 35 days in order to observe the
299 responses of G6PDH, MDA and vitamin E relative to Cd and Cu concentrations measured under
300 *in situ* conditions at the various sampling points. Statistical analyses were conducted on all
301 results obtained and are presented in the form of correlation graphs (Figs. 5-7). Although
302 biomarker responses seemed to be well correlated with Cd concentrations during the *in vitro*
303 experiments, *in situ* results considering Cd in surface waters could not be interpreted. This
304 outcome is explained by the overly weak Cd concentrations at more than 75% of the analyzed
305 sites. Consequently, no graphical depictions of correlations between biomarker responses and
306 *in situ* Cd contents are proposed.

307
308 According to the graphical representation of G6PDH enzyme activities, a positive correlation
309 is observed both for the first six days after *M. alterniflorum* introduction in the rivers and for
310 the date corresponding to 21 days (Figs. 5A-C and F). At the 21-day mark however, the ultimate
311 point at 12.6 $\mu\text{g Cu/L}$ would seem to strongly influence the p-value and hence the positive
312 correlation. Yet the compilation of all data (Fig. 5I) shows this positive correlation between Cu
313 and G6PDH activity, as supported by the Kendall test as well as the significant character ($\tau =$
314 0.39, $p = 4.37\text{E-}09$). Moreover, the set of points is more widely scattered from 0 to 6 $\mu\text{g Cu/L}$,
315 but most of them remain close to the trend line.

316
317 As for G6PDH activity, the *M. alterniflorum* MDA content appears to be positively correlated
318 with the water Cu concentration on almost all dates after plant introduction into the rivers (Figs.
319 6A, B, C, E, F, H). When assembled (Fig. 6I), the data clearly underscore this observation ($\tau =$
320 0.69, $p < 1.00\text{E-}12$), generating great support by the set of points obtained at 3, 6, 14 and 21
321 days (with respective $\tau = 0.94, 0.76, 0.76$ and 0.89), and this despite two points lying far from

322 the trend line at 35 days (1.391 and 1.204 $\mu\text{g Cu/L}$), which tend to decrease the robustness of
323 this positive correlation. Without these two values, the statistical analyses would in fact yield:
324 $\tau = 0.88$ and $p = 1.95\text{E-}03$ at 35 days, in comparison with $\tau = 0.49$ and $p = 4.43\text{E-}02$.

325

326 On the other hand, the watermilfoil vitamin E content correlation with Cu concentration in
327 rivers is not as noticeable as the previous correlation with MDA. Let's acknowledge that only
328 four dates display significant correlations (at 1, 3, 14 and 28 days in Figs. 7A, B, E and G),
329 albeit with: $-0.41 < \tau < -0.69$ and $4.718\text{E-}02 < p < 2.600\text{E-}04$. It should also be noted that the
330 21-day correlation (Fig. 7F) is not considered as a significant result since one point at 12.6 $\mu\text{g/L}$
331 among the 14 (the same Cu measurement as that commented previously for the G6PDH
332 correlation) exerts too much influence on the τ correlation coefficient and p-value ($\tau = -0.60$
333 and $p = 4.07\text{E-}03$ without this point). Interestingly however, the results in Figure 7I strongly
334 suggest a negative correlation between vitamin E content and *in situ* water Cu concentration
335 (i.e. $\tau = -0.45$ and $p = 1.06\text{E-}11$).

336

337 From all the positive and negative correlations observed and commented above, it should be
338 noted that the data obtained between 1 and 6 days after *M. alterniflorum* introduction in rivers
339 can engender a certain bias. This suggestion is dependent on the *in situ* acclimatization of plants,
340 which require a few days to stabilize their physiological parameters. Therefore, the values
341 between 1 and 6 days must not be considered independently, due to the appearance of errors,
342 yet may be used for a general data compilation approach.

343

344

345 DISCUSSION

346 This study presents three major plant biomarkers that are typically analyzed during metal stress
347 experiments. These biomarkers are actually quite sensitive to the oxidative stress produced by
348 the ROS release in the cell compartment, but not specific to TME. They may be used as
349 enzymatic or non-enzymatic indicators of biotic or abiotic stress, namely: MDA and antioxidant
350 enzymes evaluated on *Hydrilla verticillata* hydroponic cultures subjected to different pH (Song
351 *et al.*, 2018), G6PDH activity evaluated during salt stress on *Cryptocoryne elliptica* (Ahmad *et*
352 *al.*, 2017), or vitamin E evaluated during a cyanobacterial anatoxin-a stress event on
353 *Ceratophyllum demersum* (Ha *et al.*, 2014). Moreover, other biomarkers relative to plant
354 physiology or plant anatomy were previously evaluated from *in vitro* assays on this same *M.*
355 *alterniflorum* clone (Delmail *et al.*, 2011b, c). The present study focuses on two TME (Cu and
356 Cd) and just three biomarkers evaluated from both *in vitro* and *in situ* stress experiments in
357 order to carry out a comparative analysis between these two experimental systems. However,
358 some 75% of surface waters from 14 localities displayed weak Cd concentrations, thus
359 rendering any correlation analyses between this TME and biomarker responses in *M.*
360 *alterniflorum* impossible. Consequently, the comparative analysis between *in vitro* and field
361 experiments was only conducted for Cu in the part below.

362

363 G6PDH biomarker

364 During Cd or Cu stress, G6PDH activity is well known to increase, so as to compensate for the
365 deficiency in photosynthetic ATP and NADPH caused by metal stress (Honjoh *et al.*, 2003).
366 This increase had in fact already been observed for Cd stress in *Pisum sativum* (Smiri *et al.*,
367 2009) and for Cu stress in *Boerhavia diffusa*, *Tabernaemontana divaricata* (Mandal, 2006),
368 *Panax ginseng* (Ali *et al.*, 2006) and *Populus deltoides* (Lorenc-Plucińska and Stobrawa, 2005).
369 Regardless of the metal treatments, the results presented herein clearly show this increase (as

370 represented by a succession of peaks) despite the variations and fluctuations in terms of
371 biomarker response time and intensity vs. the metal concentrations employed (Figs. 3 and 4).
372 Independently of time exposure, three major activity peaks can be distinguished for all Cu
373 treatments: an initial peak appears between 1 and 3 days, followed by a second peak between 7
374 and 11 days, and lastly the final peak between 11 and 25 days. Interestingly, another peak(s) is
375 observed for 10, 25, 50 and 100 $\mu\text{g CuSO}_4/\text{L}$ treatments, with a maximum of 6 peaks at the
376 higher CuSO_4 concentration only. As Cu concentration in the medium increases, G6PDH
377 activity peaks thus seem to be more abundant and/or early, yet this correlation does not really
378 appear to be obvious given that sizable response fluctuations wind up scrambling the reading,
379 most likely as a result of several antioxidant systems triggered by ROS starting up at different
380 intensities and times. Also, most ROS are normally localized in chloroplasts (principally $^1\text{O}_2$)
381 during metal stress (Mittler, 2002; Gill and Tuteja, 2010), which mainly suggests NADPH and
382 ATP disorders in photosynthetic pathways (Nagajyoti *et al.*, 2010). As observed in Delmail *et*
383 *al.* (2011b) however, Cu stress did not significantly affect the pigment contents of *M.*
384 *alterniflorum* (as opposed to the negative effects observed for aquatic macrophytes such as
385 *Ceratophyllum demersum* (Markich *et al.*, 2006) and *Hydrilla verticillata* (Srivastava *et al.*,
386 2006). Consequently, the lack of effect on photosynthetic systems, hence the relative absence
387 of NADPH and ATP disorders, could explain the ineffective correlation between Cu
388 concentration and G6PDH activity in watermilfoil. However, since cytosolic and plastidic
389 G6PDH enzymes are governed by distinct mechanisms (Hauschild and von Schaewen, 2003),
390 the profiles obtained were perhaps the result of a combination of activity peaks from G6PDH
391 isoforms. The release of molecules by the plants in the culture medium, in response to the Cu
392 exposure (i.e. sugars) added to the abiotic stress itself, could therefore differentially activate the
393 G6PDH isoforms, which in turn would prevent detection of the direct correlation between Cu
394 and the biomarker. Similarly, activity profile analyses do not follow a logical pattern for Cd

395 treatments, yet the group of more intense peaks does seem to shift earlier relative to the increase
396 in Cd concentration. The same hypotheses as for Cu exposure can thus be suggested here.
397 In any event, even if an absence of effective correlation between Cu or Cd concentration and
398 G6PDH activity is noted for *in vitro* experiments, the *in situ* results from the 14 localities serve
399 to substantially complete this study. A positive correlation of this enzyme with Cu in the rivers
400 has in fact been highlighted (Fig. 5I). It is important to note however that other unmeasured
401 parameters (e.g. other pollutants, macronutrients, weather data) might contribute and exert an
402 additional impact on this apparent correlation, especially since it had not been observed *in vitro*.
403 Even if the Cu concentrations in rivers did not exceed 12.6 µg/L, surface waters still constitute
404 a dynamic system in which metal and metalloid concentrations are nearly maintained in spite
405 of fluctuations. Contrary to the *in vitro* assay, Cu is bioaccumulated in *Myriophyllum*
406 *alterniflorum* throughout the *in situ* experiment, which clearly explains this observed
407 correlation. It could be assumed therefore that the *M. alterniflorum* G6PDH response during
408 Cu stress is probably adjusted to the Cu concentration. G6PDH could indeed provide an
409 interesting biomarker of *in situ* Cu pollution.

410

411 **MDA biomarker**

412 The second biomarker analyzed in this study offers a good indicator of membrane
413 lipoperoxidation; it is directly related to the ROS ($O_2^{\bullet-}$, $\bullet OH$ and $\bullet OOH$) produced during metal
414 stress, as previously observed in water hyacinths (*Eichhornia crassipes*) when exposed to lead
415 (Malar *et al.*, 2016) or in the Chinese brake fern (*Pteris vittata*) and Boston fern (*Nephrolepis*
416 *exaltata*) exposed to mercury (Chen *et al.*, 2009). In this study, regardless of the metal stress
417 applied, the MDA profiles remain very similar. More specifically, the MDA content clearly
418 increases from 9 to 13 days for $CdCl_2$ concentrations above 0.5 µg/L (and from 11 to 21 days
419 for all $CuSO_4$ concentrations), followed by a slow MDA decrease until a plateau phase at 21

420 days (close to T0 for Cd treatment), then a rapid decrease and stabilization at 23 days (close to
421 T0) are observed for Cu treatments. These last phases cannot be precisely explained, however
422 two hypotheses can be proposed: (i) the MDA is more efficiently removed (since this aldehyde
423 is itself toxic for the cell); and/or (ii) one or several antioxidant systems against ROS have just
424 been implemented or are more efficient at reducing the oxidative stress.

425 Lastly, despite these similarities between profiles obtained during metal exposure, MDA
426 content seems to be correlated to the Cd concentration, although this remark is not true for Cu
427 treatments, a finding justified by an MDA content leveling off around 14 mmol MDA/g
428 watermilfoil FW regardless of CuSO₄ concentration when lying between 10 and 100 µg/L.
429 Based on this observation, it appears that a threshold limits the impact of oxidative stress,
430 suggesting the strong participation of efficient antioxidant systems. On the contrary, the *in situ*
431 experiment clearly demonstrates the positive correlation between MDA content and Cu
432 concentration, as shown in Figure 6I. Consequently, MDA could constitute an easy-to-establish
433 biomarker for water biomonitoring.

434

435 **Vitamin E**

436 Vitamin E is a liposoluble molecule localized in organelle and cell membranes, but mainly in
437 chloroplast plastoglobuli (Gill and Tuteja, 2010; Szymańska and Kruk, 2010). During metal
438 exposure, this compound allows •OH, •OOH and ¹O₂ reductions, thus detoxifying the cell
439 compartment by limiting the membrane lipoperoxidation (Gill and Tuteja, 2010). In spite of
440 antioxidant activities, only a few studies have reported α-tocopherol as an indicator of oxidative
441 stress in aquatic plants (Ha *et al.*, 2014). The results obtained here with Cu or Cd treatments
442 show similar profiles primarily characterized by a decrease in vitamin E content after 7 to 11
443 days of metal exposure, followed by a rapid increase until the level observed at T0. In addition,
444 for Cd stress, the vitamin E amount appears to be clearly dependent on the contaminant

445 concentration, except at the lower concentration of 0.5 $\mu\text{g/L}$ CdCl_2 . For this pollutant therefore,
446 the oxidative stress on chloroplast and membranes is quite high after the first week of metal
447 exposure. Afterwards, this effect is reversed (vitamin E increase), leading to assume that plants
448 cope with this stress by using other reducing agents or by *de novo* vitamin E synthesis. In either
449 case (Cu or Cd), the restoration of vitamin E content to its basal level between the 2nd and 3rd
450 week is not due to a decrease in ROS pressure, given that the curves depicting values of metal
451 concentration accumulated in plants are practically linear during this period (Fig. 2).

452 As mentioned above, the membrane lipoperoxidation (MDA profiles) is observed between
453 Days 7 and 9 (Cd exposure) or between Days 11 and 13 (Cu exposure), matching the vitamin
454 E decrease. This lipoperoxidation maximum and vitamin E content minimum are reached
455 simultaneously at 13 days for an exposure between 1 and 10 $\mu\text{g CdCl}_2/\text{L}$. Beyond 13 days,
456 curve inversions were observed, thus indicating reduced lipoperoxidation during Cd exposure.
457 Altogether, these results suggest that the lipoperoxidation determines the vitamin E content and
458 moreover vitamin E degradation during the peroxidation of lipids occurs to a great extent after
459 one week of Cd treatment. On the other hand, a weak and limited MDA response was observed
460 regardless of Cu concentration and in spite of a very slight and insignificant vitamin E decrease
461 observed between Days 11 and 21. Nevertheless, the α -tocopherol response to Cu treatment
462 seems to be correlated with the MDA profile as well, even though this compound is not
463 significantly correlated with Cu concentration. Consequently, since the oxidative stress appears
464 to reach a maximum during Cu exposure, it could be assumed that a process is restraining a key
465 factor required for ROS production. The elimination of intracellular Cu^{2+} by another
466 detoxification pathway, such as phytochelatinases, could substantiate this hypothesis. These
467 chelating agents may indeed be involved in the partial sequestration of divalent ions, which are
468 essential to ROS production (Iturbe-Ormaetxe *et al.*, 1995; Ranieri *et al.*, 2001; Tewari *et al.*,
469 2005; Salama *et al.*, 2009). The oxidative stress threshold is also influenced by the absence of

470 a significant negative impact on the photosynthetic pathway since pigment contents were not
471 impaired (Delmail *et al.*, 2011b).

472 Lastly, the significant negative correlation (Fig. 7I) in the *in situ* experiment is not as robust as
473 that observed for MDA. After 5 μg Cu/L in water, the dots become more scattered, meaning
474 that this type of experiment requires more data from rivers contaminated with higher Cu
475 concentrations. Nevertheless, the vitamin E decrease in response to *in situ* Cu contamination is
476 in agreement with the negative correlation between vitamin E content and Cu in *Pinus sylvestris*
477 growing at TME-contaminated sites (Pukacki and Kamińska-Rozek, 2002). As observed in
478 these preliminary data, vitamin E content measured from the macrophyte *Myriophyllum*
479 *alterniflorum* and used as a marker of Cu pollution in rivers could constitute a new and
480 innovative tool for *in situ* biomonitoring.

481

482

483 CONCLUSION

484 As opposed to other datasets focused over short period analyses, the results provided herein
485 constitute a broad and substantial study since the assays were performed *in vitro* and *in situ*
486 covering an extensive period and at high frequency. Unfortunately, divergences in results
487 appear between the two experimental systems since the biomarker responses of G6PDH, MDA
488 and vitamin E only seemed to be correlated with the *in situ* Cu exposure. Analyses of these
489 results thus suggest the use of *Myriophyllum alterniflorum* vitroplants and these biomarkers for
490 water biomonitoring, although this suggestion is not readily apparent from the *in vitro* results.
491 The fact of introducing just a single supplementation to the synthetic culture medium at the
492 beginning of the experiment could partially explain the results obtained. The pollutant is being
493 quickly bioaccumulated *in vitro*, while the *in situ* surface waters almost permanently maintain
494 the metal concentration. This difference is probably due to the absence of any analogy between

495 the two experimental systems. It also appears to be of paramount importance to complete and
496 upgrade the field experiments with data obtained in rivers presenting high Cu content, since
497 most Cu concentrations at the various tested sites were less than 5 µg/L. Similarly, no
498 correlation analyses from field assays could be conducted between these biomarkers and Cd
499 given that too few localities exhibited sufficient contamination levels for this TME. In addition,
500 since field data are dependent on multiple parameters, not all of which are measurable, the
501 positive correlations observed must be tempered. It is essential to note that these *in situ* results
502 are preliminary as only metal contamination by Cu or Cd was considered. For example, let's
503 not overlook the fact that organic or inorganic pollutants not analyzed in this study definitely
504 exert an impact on the biomarker responses, thus relegating the conclusions proposed here to
505 simply a set of hypotheses. Consequently, supplementary experiments that consider other
506 physicochemical parameters and multi-elemental contaminations would need to be performed.
507 Moreover, omics and principal component analyses would be of great interest in the detection
508 of biomarkers specific to metal pollution. Such improvements would definitively validate the
509 use of this macrophyte as an early and robust bioindicator of water contamination by trace metal
510 elements.

511

512

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689

690

691 **FIGURES**

692 Figure 1: Map of the 14 *in situ* localities (loc.) where 35-day acclimatized *Myriophyllum*
693 *alterniflorum* plants were deployed. (A) Localization of the geographic study zone on a map of
694 France (red circle). (B) Enlargement of the circled red zone observed in A. River names are
695 surrounded by rectangles; the villages, towns and city (Limoges) are written in italics. (C) GPS
696 coordinates of the various localities, as measured with Multi Navigator (Silva). Each locality is
697 depicted by a number placed on the map in (B), and a brief description of each position is
698 provided. Downstream (downstr.) and upstream (upstr.) indicate that the water was sampled at
699 this particular position from the designated locality.

700

701 Figure 2: Bioaccumulation of Cu or Cd in 500 mg of dry leaves (DW) of *Myriophyllum*
702 *alterniflorum* treated with 100 µg/L CuSO₄ (A) or 10 µg/L CdCl₂ (B) for 27 days. Each
703 histogram is a triplicate mean; the vertical lines above the bars indicate standard deviations.

704

705 Figure 3: Impact of various Cu concentrations on *Myriophyllum alterniflorum* biomarkers. Five
706 concentrations of CuSO₄ were used and supplemented (5, 10, 25, 50, 100 µg/L) *in vitro* in the
707 synthetic oligotrophic medium. The kinetic responses of three biomarkers were analyzed over
708 27 days: G6PDH, MDA, and vitamin E. Each histogram bar corresponds to a three-technical
709 replicate mean, and the error bars indicate standard deviations (for each biomarker and in each
710 time frame, three independent clones were ground together).

711

712 Figure 4: Impact of various Cd concentrations on *Myriophyllum alterniflorum* biomarkers. Five
713 concentrations of CdCl₂ were used and supplemented (0.5, 1, 4, 7, 10 µg/L) *in vitro* in the
714 synthetic oligotrophic medium. The kinetic responses of three biomarkers were analyzed over

715 27 days: G6PDH, MDA, and vitamin E. Each histogram bar corresponds to a three-technical
716 replicate mean, and the error bars indicate standard deviations (for each biomarker and in each
717 time frame, three independent clones were ground together).

718

719 Figure 5: Graphical representation of the correlation between *Myriophyllum alterniflorum*
720 G6PDH activity and Cu concentration measured in water at 14 different *in situ* sites after: 1 day
721 (A), 3 days (B), 6 days (C), 8 days (D), 14 days (E), 21 days (F), 28 days (G), and 35 days (H).
722 (I) corresponds to the compilation of all 5-week data obtained across all sites. On each graph,
723 the τ Kendall's correlation value and corresponding p-value are indicated. All data correspond
724 to triplicate, but only the mean without standard deviation is shown in order to avoid
725 overloading the figure.

726

727 Figure 6: Graphical representation of the correlation between *Myriophyllum alterniflorum*
728 MDA content and Cu concentration measured in water at 14 different *in situ* sites after: 1 day
729 (A), 3 days (B), 6 days (C), 8 days (D), 14 days (E), 21 days (F), 28 days (G), and 35 days (H).
730 (I) corresponds to the compilation of all 5-week data obtained across all sites. On each graph,
731 the τ Kendall's correlation value and corresponding p-value are indicated. All data correspond
732 to triplicate, but only the mean without standard deviation is shown in order to avoid
733 overloading the figure.

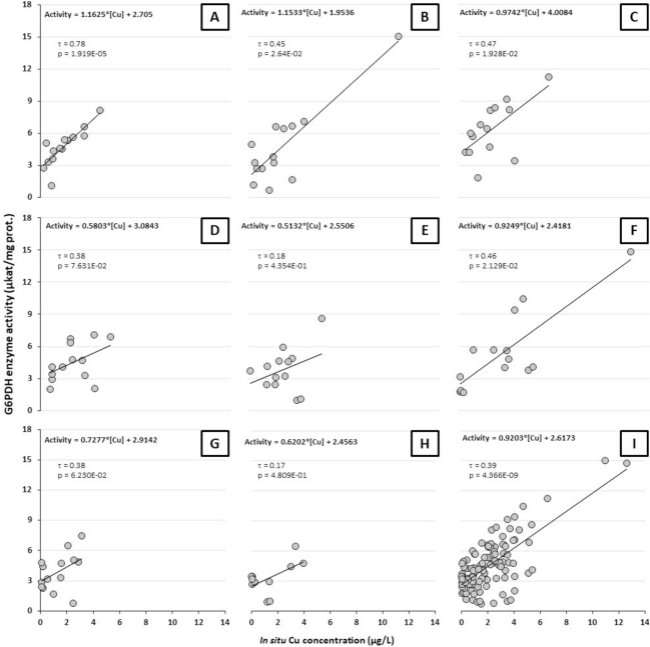
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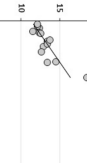
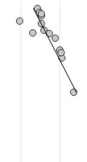
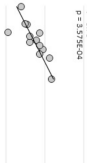
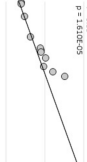
735 Figure 7: Graphical representation of the correlation between *Myriophyllum alterniflorum*
736 vitamin E content and Cu concentration measured in water at 14 different *in situ* sites after: 1
737 day (A), 3 days (B), 6 days (C), 8 days (D), 14 days (E), 21 days (F), 28 days (G), and 35 days
738 (H). (I) corresponds to the compilation of all 5-week data obtained across all sites. On each
739 graph, the τ Kendall's correlation value and corresponding p-value are indicated. All data

740 correspond to triplicate, but only the mean without standard deviation is shown in order to avoid
741 overloading the figure.

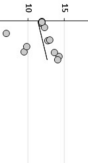
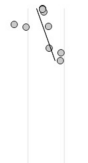
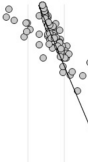
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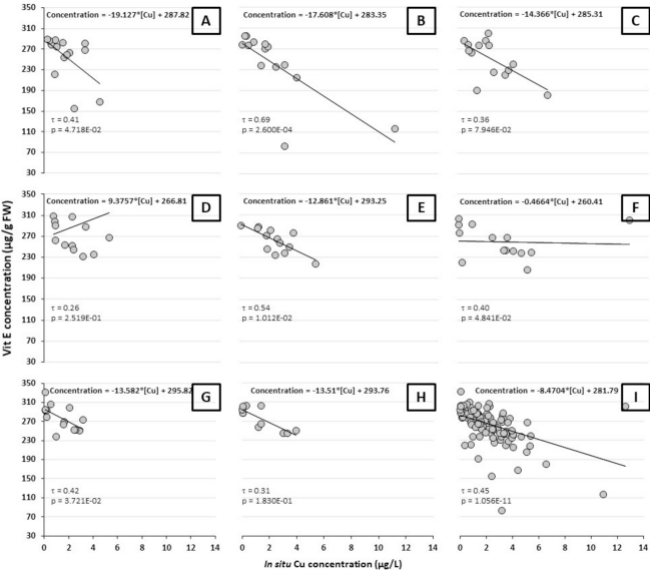
- Watermilfoil is evaluated through biomarkers for its use in water quality monitoring.
- G6PDH, MDA and VitE biomarkers are not correlated with *in vitro* Cu exposures.
- Only MDA and VitE biomarkers are correlated to Cd concentration *in vitro*.
- Interestingly, correlations between freshwater [Cu] and all biomarkers are evidenced.
- Other field parameters need to be considered for the evaluation of this plant model.



AConcentration = $1.1415^{\text{[Cu]}} + 11.502$ $r = 0.66$ $p = 1.962\text{E-}03$ **B**Concentration = $0.9193^{\text{[Cu]}} - 11.54$ $r = 0.94$ $p = 3.949\text{E-}06$ **C**Concentration = $0.901^{\text{[Cu]}} + 11.243$ $r = 0.76$ $p = 1.816\text{E-}04$ **D**Concentration = $0.3844^{\text{[Cu]}} + 12.11$ $r = 0.29$ $p = 1.179\text{E-}01$ **E**Concentration = $0.6341^{\text{[Cu]}} + 11.675$ $r = 0.76$ $p = 3.575\text{E-}04$ **F**Concentration = $0.6341^{\text{[Cu]}} + 11.675$ $r = 0.89$ $p = 1.610\text{E-}05$ 

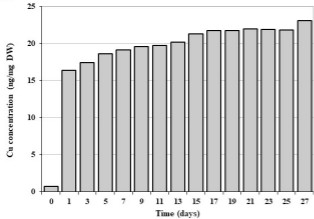
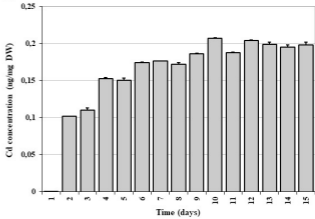
MDA concentration (nmol/g FW)

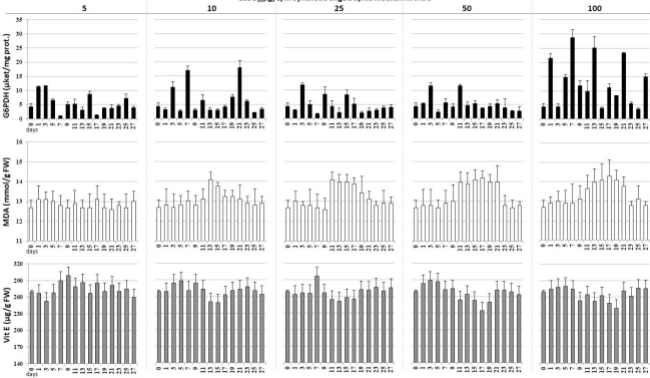
GConcentration = $0.406^{\text{[Cu]}} + 11.319$ $r = 0.36$ $p = 8.129\text{E-}02$ **H**Concentration = $0.63^{\text{[Cu]}} - 11.176$ $r = 0.49$ $p = 4.439\text{E-}02$ **I**Concentration = $0.764^{\text{[Cu]}} + 11.432$ $r = 0.69$ $p = 0.000$ *In situ* Cu concentration (µg/l)





- C**
- | | | |
|--|---|---|
| -loc.1: 45°47'81"N 1°08'61"E → Aureance and Vienne confluence (downstr.) | -loc.2: 45°49'29"N 1°12'39"E → close to the road | -loc.3: 45°52'86"N 1°16'77"E → industrial zone |
| -loc.4: 45°54'73"N 1°16'13"E → farming zone (downstr.) | -loc.5: 45°54'28"N 1°13'25"E → Aureance's source | -loc.6: 45°47'49"N 1°13'24"E → tannery waste (downstr.) |
| -loc.7: 45°47'94"N 1°13'70"E → Valoine and Vienne confluence (downstr.) | -loc.8: 45°49'04"N 1°15'36"E → water treatment plant (downstr.) | -loc.9: 45°50'10"N 1°28'49"E → villager zone |
| -loc.10: 45°47'16"N 1°33'19"E → Combade and Vienne confluence (downstr.) | -loc.11: 45°51'71"N 1°19'39"E → smelting plant (downstr.) | -loc.12: 45°49'05"N 1°15'10"E → junkyard (downstr.) |
| -loc.13: 45°48'23"N 1°15'50"E → urban area (downstr.) | -loc.14: 45°48'10"N 1°17'07"E → industrial zone exposed also to expressway contamination (upstr.) | |

A**B**

CuSO₄ (μg/L) in synthetic oligotrophic medium *in vitro*

CdCl₂ (μg/L) in synthetic oligotrophic medium *in vitro*