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PII: S0045-6535(19)30126-2
DOI: 10.1016/j.chemosphere.2019.01.105
Reference: CHEM 23020
To appear in: Chemosphere

Received Date: 19 September 2018
Accepted Date: 20 January 2019

Please cite this article as: Raphaël Decou, Servane Bigot, Philippe Hourdin, David Delmail, Pascal Labrousse, Comparative in vitro/in situ approaches to three biomarker responses of Myriophyllum alterniflorum exposed to metal stress, Chemosphere (2019), doi: 10.1016/j.chemosphere.2019.01.105

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

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**ABSTRACT**

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

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

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**INTRODUCTION**

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

Contrary to most organic pollutants, TME do not degrade in surface waters, sediments or soil whenever long removal processes are involved, thus resulting in long-term impacts on
ecological communities (Hayes et al., 2018). Depending on the TME under consideration, the effects on organisms differ according to the concentration, chemical structure, bioavailability and utility of the particular TME in biological processes. For example, at low concentrations, copper (Cu) is an essential compound involved in many metabolic pathways, while cadmium (Cd) is considered to be non-essential and toxic for the biological development of organisms by virtue of inhibiting various processes in plant metabolism (Delmail et al., 2011b, 2011c). Low Cd concentrations however may be beneficial, as observed for example on Miscanthus sinensis (Arduini et al., 2004) and Triticum aestivum growths (Lin et al., 2007). In any case, all TME, whether essential or not, tend to be toxic at high concentrations and accumulate in organisms.

The increase of TME concentrations in ecosystems over the past few decades, as observed in the food chain (Zhang et al., 2017), has led to establishing new regulations to better protect both the environment and biodiversity. Along these lines, the European Water Framework Directive (2000/60/EC) was instituted in 2000 to maintain or restore the ecological and chemical qualities of surface waters and groundwater. Accordingly, such objectives require constantly evaluating water quality by means of biomonitoring. Fish, mollusks and invertebrates are extensively used as bioindicators of metal pollution (Van Praet et al., 2014; Wang and Lu, 2017; Bouzahouane et al., 2018; Souza et al., 2018), although aquatic plants are also increasingly being investigated for these ecological surveys (Ferrat et al., 2003; Krayem et al., 2016; Reale et al., 2016). More specifically, the analyses of association, composition and richness of plant species during in situ studies have become even more widespread and in depth. In addition to the level of metal accumulation in organisms, the physiological, biochemical and anatomical changes in organs or in the entire organism have for a number of years provided an interesting way forward in the field of water biomonitoring.
Myriophyllum L. (Haloragaceae R.By.), a cosmopolitan and submerged aquatic macrophyte genus comprising some 60 species (Lü et al., 2017), is able to accumulate chemicals, as observed in several studies, i.e. in situ accumulation of cobalt, lead, zinc, nickel, iron and manganese in Myriophyllum aquaticum (Harguinteguy et al., 2013, 2016) or in Myriophyllum spicatum (Galal and Shehata, 2014). Cu, Cd or arsenic hyperaccumulation in Myriophyllum alterniflorum has also been evaluated from in vitro cultures (Delmail et al., 2011b, 2011c; 2013; Krayem et al., 2016; Krayem et al., 2018). In these last studies however and in contrast with other studies, biological changes were also measured through biomarker response analyses focusing on scavengers indicating the oxidative stress generated by reactive oxygen species (ROS) and/or antioxidant systems.

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

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

**MATERIALS AND METHODS**

**Micropropagation and plant growth**

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

**\textit{In vitro} assays in artificial media supplemented by copper or cadmium**

After 35 days, plant clones were acclimatized over 2 weeks in a new medium with a chemical composition identical to the Vienne River (i.e. oligotrophic medium), as described in Delmail \textit{et al.} (2011a). 450 clones were thus transferred into culture boxes containing 300 mL of oligotrophic medium (apportioned 10 explants per box). CuSO\(_4\), 5 H\(_2\)O or CdCl\(_2\), 2.5 H\(_2\)O
(VWR, USA) was added aseptically to the medium in 15 boxes (for each tested concentration) in order to obtain a final concentration ranging either from 5 to 100 µg CuSO$_4$/L (corresponding to 1.3 - 25.5 µg Cu/L) or from 0.5 to 10 µg CdCl$_2$/L (corresponding to 0.2 - 4.9 µg Cd/L). These concentrations were chosen for their similarity to those observed in the Vienne River (0.5 - 12.6 µg Cu/L and 0.04 - 1.4 µg Cd/L). The other 15 boxes served as controls. The experiment was performed for 27 days, and biological measurements were recorded every other day.

**In situ plant deployment and sampling**

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

**Protein extraction and quantification**

For the *M. alterniflorum* enzyme activities presented below, protein extractions were performed according to a method based on the protocol developed by Srivastava *et al.* (2006). Fresh plant material (200 mg) was ground at +4°C in a 800-µL buffer containing 50 mM Tris-HCl (pH 7.0), 3.75% polyvinyl pyrrolidone (PVP) (w/v), 0.1 mM ethylenediaminetetraacetic acid
(EDTA) and 1 mM phenylmethylsulfonyl fluoride (PMSF). All these compounds were obtained from Sigma-Aldrich (USA). The extracts were centrifuged at 10,000 g for 10 min at +4°C (3-18K, Sigma-Aldrich, USA), and the total protein contents were quantified using the Bradford assay (Bradford, 1976). 50 µL of protein extract were added to a cuvette containing 1.5 mL of Bradford Reagent (Sigma-Aldrich, USA). After a 15-min incubation at room temperature, the absorbance of the protein-dye complex was read at 595 nm (Heλios Beta, Thermo Spectronic, USA). The protein concentrations were determined by comparison with the standard curve prepared using the protein standard, i.e. bovine serum albumin (BSA, Sigma-Aldrich).

**Watermilfoil enzyme activity**

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

**Estimation of lipoperoxidation**

Malondialdehyde (MDA) quantification is based on the method derived by Heath and Packer (1968). 250 mg of three watermilfoil clones (apex) were ground at +4°C with a pestle in a
mortar containing 1 g of Fontainebleau sand and 12.5 mL of 1% trichloroacetic acid (m/v; TCA, Sigma-Aldrich, USA). The extract was then centrifuged at 2,600 g for 10 min (3-18K, Sigma-Aldrich, USA). One mL of the supernatant was added to 1 mL of 0.5% thiobarbituric acid (TBA; prepared in 20% TCA, m/v, Sigma-Aldrich, USA), and the mix was boiled at +100°C for 30 min in a water bath before being chilled on ice for 5 min. Next, the samples underwent 10 min of 2,600-g centrifugation at +4°C, and the absorbance was read at 532 nm in a spectrophotometer (Helios Beta, Thermo Spectronic, USA). The MDA concentration was calculated using both the molar extinction coefficient (i.e. 1.55E-3 cm²·mmol⁻¹) and Beer-Lambert Law (Beer, 1852); it was expressed in mmol/g FW.

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

Trace metal elements in water and plants
The copper and cadmium contained in the samples were measured in triplicate using ICP-MS (SCIEX-ELAN 6100 DRC, Perkin-Elmer, USA) from both *in vitro* collected *M. alterniflorum* and freshly sampled waters (for each locality, water was sampled once on Days 1, 3, 6, 8, 14, 21, 28 and 35 after plant deployment in the rivers). For plant mineralization, watermilfoil clones were carefully rinsed in running tap water, thoroughly washed with distilled water and blotted dry; 500 mg of fresh weight were digested in 2.5 mL HNO₃ 69% (Fisher chemical, Thermo Fisher Scientific, USA) at 60°C for 48 h. 500 microliters of the solution were diluted in 2 mL of aqueous solution (4.5‰ n-butanol, 0.1 g/L ammonia, 0.1 g/L EDTA, 1‰ germanium as the...
internal standard) and then introduced into the nebulizer. Five-point reference scales were
applied according to the same protocol as before (from 0.1 to 10 µg/L Cd and 0.5 to 50 µg/L
Cu). For each sample, five technical replicates were performed. The limits of quantification
(LQ) were: 0.1 µg/L for Cd, and 0.5 µg/L for Cu. The Plasma Power, Plasma Gas Flow and
Auxiliary Gas Flow equaled 1,350 W, 15 L/min and 1.175 L/min, respectively.

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

RESULTS

*M. alterniflorum* biomarker response to Cu
During the initial hours of 100 µg/L CuSO\(_4\) treatment, *M. alterniflorum* accumulated Cu very
quickly (as the watermilfoil Cu content increased by a factor of 21.6 during the first day, i.e.
from 0.76 to 16.4 ng Cu/mg Dry Weight (DW), see Fig. 2A). This accumulation was also
observed for lower-level CuSO\(_4\) treatments (data not shown). Beyond this initial period,
between 1 and 27 days of treatment, the Cu phytoaccumulation increased slowly, with an
apparent stabilization around 22 ng Cu/mg DW during the last 10 days of analysis. Due to these
homogeneous Cu bioaccumulations from Days 1 through 27, the origins of heterogeneous differences in biomarker profiles were difficult to define (Fig. 3).

The entire G6PDH profiles remained moderately homogeneous throughout the assay yet by focusing on a shorter period (from 3 to 15 days), greater profile homogeneity was observed. During this period, the maximum homogeneity was obtained not only for 5 and 25 µg CuSO₄/L but also for 50 µg CuSO₄/L. For these particular activity profiles, the relative homogeneity can be demonstrated through similar peaks: initial peak at 3 days (+168.9%, +309.9% and +167.4%, respectively - Note: The percentage values expressed here correspond to the total increase between peak beginning and peak end); second peak at 11 days (+367.8%), 9 days (+425.4%) and 7 days (+132.1%); and third peak at 15 days (+178.4% and +302.0%) and 11 days (+173.1%). In contrast, the activity profiles at 10 and 100 µg CuSO₄/L shared more similarities during the final 13 days of analysis: i.e. one peak (+508.7%) at 21 days for 10 µg CuSO₄/L and in the form of 2 peaks (+194.4% at 17 days and +183.5% at 21 days) for 100 µg CuSO₄/L; another peak at 27 days (+55.0% and +332.9%) for 10 and 100 µg CuSO₄/L, respectively.

According to the MDA profiles (Fig. 3), biomarker responses increased between Days 11 and 21 for all CuSO₄ concentrations (except 5 µg CuSO₄/L). Although the MDA level seems to be higher in response to the increase of Cu in the medium, no apparent correlation between these two parameters could be detected. The maximum increase for each treatment was: +3.4% at 17 days, +10.1% at 13 days, +12.0% at 11 days, +11.9% at 17 days, and +10.9% at 17 days for 5, 10, 25, 50 and 100 µg CuSO₄/L, respectively. Beyond this period, the MDA content decrease was measured as of Day 23.
*M. alterniflorum* vitamin E content in response to various CuSO₄ concentrations revealed slightly similar profiles during the 27-day treatment (Fig. 3), which indicates the lack of correlation between vitamin E level and Cu concentration. All profiles showed a sharp increase in vitamin E during the first few days, appearing earlier for high Cu concentrations (compared to the control level: +18.9% at 9 days, +7.2% and +4.9% at 5 and 9 days (two adjacent peaks), +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, respectively). Interestingly, at around 15 ± 2 days, a distinct yet rather weak decrease in vitamin E could be observed for all profiles (except at 5 µg CuSO₄/L), followed by a plateau phase occurring later for high Cu concentrations (at 17, 19 and 21 days for 10, 25 and 50-100 µg CuSO₄/L, respectively).

*Myriophyllum alterniflorum* biomarker response to Cd

Similar to the previous comments on Cu phytoaccumulation in *M. alterniflorum*, Cd accumulation presented similar profiles during the 27 days of 10 µg/L CdCl₂ treatment (Fig. 2B). By the day after treatment, the clones had absorbed 0.1 ng Cd/mg DW, and this value reached 0.21 ng Cd/mg DW at 17 days. A plateau phase was observed next. For the other CdCl₂ concentrations used as treatments in the synthetic media, the Cd accumulation profiles were analogous to the previous one, but with the plateau phase appearing more quickly (data not shown). Once again, like for the Cu treatment presented above, this fast Cd bioaccumulation in plants complicates the definition of biomarker response origins during the 1 to 27-day period (Fig. 4). Nevertheless, early biomarker responses due to these quick TME accumulations could be expected. It therefore seems important to focus on shorter time segments to explain the homogeneity and heterogeneity zones between the five response profiles of each biomarker.
Regarding the G6PDH profiles in Figure 4, it appeared to be difficult at first to find homogeneous responses between each CdCl$_2$ treatment even though 1 and 4 µg CdCl$_2$/L treatments were slightly similar over the final 7 days (respectively one peak at 21 days: +472.1% and +226.9%, and another peak at 27 days: +229.3% and +785.0%). According to biomarker response intensities over the period from Day 0 to Day 13, an analogy can be observed between 0.5 and 10 µg CdCl$_2$/L treatments as well (+253.1% at 5 days and +131.8% at 1 day / +263.8% and +190.6% at 9 days, respectively). On the contrary, the 7 µg CdCl$_2$/L profile reveals greater peak intensities (+1,029.6% at 5 days and +563.0% at 13 days).

The MDA profiles graphically indicate a biomarker response clearly sensitive to the Cd concentration between Days 11 and 17 (Fig. 4). In addition to this observation, the biomarker response is dependent on Cd concentration. The maximum MDA content actually equals: 14.73 ± 0.19, 15.7 ± 0.37, 16.45 ± 0.0, and 17.85 ± 0.37 mmol/g FW from 1 to 10 µg CdCl$_2$/L. It is interesting to note that all these values are obtained at 13 days for practically every MDA profile.

Like with the MDA biomarker, vitamin E content is also dependent on Cd concentration, although it decreased between 9 and 19 days with a minimum reached at 13 days for all CdCl$_2$ concentrations except 0.5 µg/L (Fig. 4). At this latter concentration, the vitamin E content profile in *M. alterniflorum* completely differs from the others. This result suggests that the low Cd supplementation in the oligotrophic medium did not significantly influence the biomarker response.

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

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

As for G6PDH activity, the M. alterniflorum MDA content appears to be positively correlated with the water Cu concentration on almost all dates after plant introduction into the rivers (Figs. 6A, B, C, E, F, H). When assembled (Fig. 6I), the data clearly underscore this observation (τ = 0.69, p < 1.00E-12), generating great support by the set of points obtained at 3, 6, 14 and 21 days (with respective τ = 0.94, 0.76, 0.76 and 0.89), and this despite two points lying far from
the trend line at 35 days (1.391 and 1.204 µg Cu/L), which tend to decrease the robustness of this positive correlation. Without these two values, the statistical analyses would in fact yield: τ = 0.88 and p = 1.95E-03 at 35 days, in comparison with τ = 0.49 and p = 4.43E-02.

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

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

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

G6PDH biomarker

During Cd or Cu stress, G6PDH activity is well known to increase, so as to compensate for the deficiency in photosynthetic ATP and NADPH caused by metal stress (Honjoh *et al.*, 2003). This increase had in fact already been observed for Cd stress in *Pisum sativum* (Smiri *et al.*, 2009) and for Cu stress in *Boerhavia diffusa, Tabernaemontana divaricata* (Mandal, 2006), *Panax ginseng* (Ali *et al.*, 2006) and *Populus deltoides* (Lorenc-Plucińska and Stobrawa, 2005). Regardless of the metal treatments, the results presented herein clearly show this increase (as
represented by a succession of peaks) despite the variations and fluctuations in terms of biomarker response time and intensity vs. the metal concentrations employed (Figs. 3 and 4).

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

In any event, even if an absence of effective correlation between Cu or Cd concentration and G6PDH activity is noted for in vitro experiments, the in situ results from the 14 localities serve to substantially complete this study. A positive correlation of this enzyme with Cu in the rivers has in fact been highlighted (Fig. 5I). It is important to note however that other unmeasured parameters (e.g. other pollutants, macronutrients, weather data) might contribute and exert an additional impact on this apparent correlation, especially since it had not been observed in vitro.

Even if the Cu concentrations in rivers did not exceed 12.6 µg/L, surface waters still constitute a dynamic system in which metal and metalloid concentrations are nearly maintained in spite of fluctuations. Contrary to the in vitro assay, Cu is bioaccumulated in Myriophyllum alterniflorum throughout the in situ experiment, which clearly explains this observed correlation. It could be assumed therefore that the M. alterniflorum G6PDH response during Cu stress is probably adjusted to the Cu concentration. G6PDH could indeed provide an interesting biomarker of in situ Cu pollution.

**MDA biomarker**

The second biomarker analyzed in this study offers a good indicator of membrane lipoperoxidation; it is directly related to the ROS (O$_2^*$; *OH and *OOH) produced during metal stress, as previously observed in water hyacinths (Eichhornia crassipes) when exposed to lead (Malar et al., 2016) or in the Chinese brake fern (Pteris vittata) and Boston fern (Nephrolepis exaltata) exposed to mercury (Chen et al., 2009). In this study, regardless of the metal stress applied, the MDA profiles remain very similar. More specifically, the MDA content clearly increases from 9 to 13 days for CdCl$_2$ concentrations above 0.5 µg/L (and from 11 to 21 days for all CuSO$_4$ concentrations), followed by a slow MDA decrease until a plateau phase at 21
days (close to T0 for Cd treatment), then a rapid decrease and stabilization at 23 days (close to T0) are observed for Cu treatments. These last phases cannot be precisely explained, however two hypotheses can be proposed: (i) the MDA is more efficiently removed (since this aldehyde is itself toxic for the cell); and/or (ii) one or several antioxidant systems against ROS have just been implemented or are more efficient at reducing the oxidative stress.

Lastly, despite these similarities between profiles obtained during metal exposure, MDA content seems to be correlated to the Cd concentration, although this remark is not true for Cu treatments, a finding justified by an MDA content leveling off around 14 mmol MDA/g watermilfoil FW regardless of CuSO$_4$ concentration when lying between 10 and 100 µg/L. Based on this observation, it appears that a threshold limits the impact of oxidative stress, suggesting the strong participation of efficient antioxidant systems. On the contrary, the in situ experiment clearly demonstrates the positive correlation between MDA content and Cu concentration, as shown in Figure 6I. Consequently, MDA could constitute an easy-to-establish biomarker for water biomonitoring.

**Vitamin E**

Vitamin E is a liposoluble molecule localized in organelle and cell membranes, but mainly in chloroplast plastoglobuli (Gill and Tuteja, 2010; Szymańska and Kruk, 2010). During metal exposure, this compound allows $^{\bullet}$OH, $^{\bullet}$OOH and $^{1}$O$_2$ reductions, thus detoxifying the cell compartment by limiting the membrane lipoperoxidation (Gill and Tuteja, 2010). In spite of antioxidant activities, only a few studies have reported $\alpha$-tocopherol as an indicator of oxidative stress in aquatic plants (Ha et al., 2014). The results obtained here with Cu or Cd treatments show similar profiles primarily characterized by a decrease in vitamin E content after 7 to 11 days of metal exposure, followed by a rapid increase until the level observed at T0. In addition, for Cd stress, the vitamin E amount appears to be clearly dependent on the contaminant
concentration, except at the lower concentration of 0.5 µg/L CdCl$_2$. For this pollutant therefore, the oxidative stress on chloroplast and membranes is quite high after the first week of metal exposure. Afterwards, this effect is reversed (vitamin E increase), leading to assume that plants cope with this stress by using other reducing agents or by *de novo* vitamin E synthesis. In either case (Cu or Cd), the restoration of vitamin E content to its basal level between the 2$^{nd}$ and 3$^{rd}$ week is not due to a decrease in ROS pressure, given that the curves depicting values of metal concentration accumulated in plants are practically linear during this period (Fig. 2).

As mentioned above, the membrane lipoperoxidation (MDA profiles) is observed between Days 7 and 9 (Cd exposure) or between Days 11 and 13 (Cu exposure), matching the vitamin E decrease. This lipoperoxidation maximum and vitamin E content minimum are reached simultaneously at 13 days for an exposure between 1 and 10 µg CdCl$_2$/L. Beyond 13 days, curve inversions were observed, thus indicating reduced lipoperoxidation during Cd exposure. Altogether, these results suggest that the lipoperoxidation determines the vitamin E content and moreover vitamin E degradation during the peroxidation of lipids occurs to a great extent after one week of Cd treatment. On the other hand, a weak and limited MDA response was observed regardless of Cu concentration and in spite of a very slight and insignificant vitamin E decrease observed between Days 11 and 21. Nevertheless, the α-tocopherol response to Cu treatment seems to be correlated with the MDA profile as well, even though this compound is not significantly correlated with Cu concentration. Consequently, since the oxidative stress appears to reach a maximum during Cu exposure, it could be assumed that a process is restraining a key factor required for ROS production. The elimination of intracellular Cu$^{2+}$ by another detoxification pathway, such as phytochelatines, could substantiate this hypothesis. These chelating agents may indeed be involved in the partial sequestration of divalent ions, which are essential to ROS production (Iturbe-Ormaetxe *et al.*, 1995; Ranieri *et al.*, 2001; Tewari *et al.*, 2005; Salama *et al.*, 2009). The oxidative stress threshold is also influenced by the absence of
a significant negative impact on the photosynthetic pathway since pigment contents were not impaired (Delmail et al., 2011b).

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

CONCLUSION

As opposed to other datasets focused over short period analyses, the results provided herein constitute a broad and substantial study since the assays were performed in vitro and in situ covering an extensive period and at high frequency. Unfortunately, divergences in results appear between the two experimental systems since the biomarker responses of G6PDH, MDA and vitamin E only seemed to be correlated with the in situ Cu exposure. Analyses of these results thus suggest the use of Myriophyllum alterniflorum vitroplants and these biomarkers for water biomonitoring, although this suggestion is not readily apparent from the in vitro results. The fact of introducing just a single supplementation to the synthetic culture medium at the beginning of the experiment could partially explain the results obtained. The pollutant is being quickly bioaccumulated in vitro, while the in situ surface waters almost permanently maintain the metal concentration. This difference is probably due to the absence of any analogy between
the two experimental systems. It also appears to be of paramount importance to complete and upgrade the field experiments with data obtained in rivers presenting high Cu content, since most Cu concentrations at the various tested sites were less than 5 µg/L. Similarly, no correlation analyses from field assays could be conducted between these biomarkers and Cd given that too few localities exhibited sufficient contamination levels for this TME. In addition, since field data are dependent on multiple parameters, not all of which are measurable, the positive correlations observed must be tempered. It is essential to note that these in situ results are preliminary as only metal contamination by Cu or Cd was considered. For example, let's not overlook the fact that organic or inorganic pollutants not analyzed in this study definitely exert an impact on the biomarker responses, thus relegating the conclusions proposed here to simply a set of hypotheses. Consequently, supplementary experiments that consider other physicochemical parameters and multi-elemental contaminations would need to be performed. Moreover, omics and principal component analyses would be of great interest in the detection of biomarkers specific to metal pollution. Such improvements would definitively validate the use of this macrophyte as an early and robust bioindicator of water contamination by trace metal elements.

ACKNOWLEDGMENTS

This research was financed by three sources: the European Union through the FEDER-Plan Loire project MACROPOD 2 2016EX-000856 for R. Decou's post-doctoral fellowship; the Limousin Regional Council for D. Delmail's Ph.D. thesis; and the PEREINE Laboratory EA7500 (ex-GRESE EA 4330).
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FIGURES

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

Figure 2: Bioaccumulation of Cu or Cd in 500 mg of dry leaves (DW) of Myriophyllum alterniflorum treated with 100 µg/L CuSO$_4$ (A) or 10 µg/L CdCl$_2$ (B) for 27 days. Each histogram is a triplicate mean; the vertical lines above the bars indicate standard deviations.

Figure 3: Impact of various Cu concentrations on Myriophyllum alterniflorum biomarkers. Five concentrations of CuSO$_4$ were used and supplemented (5, 10, 25, 50, 100 µg/L) in vitro in the synthetic oligotrophic medium. The kinetic responses of three biomarkers were analyzed over 27 days: G6PDH, MDA, and vitamin E. Each histogram bar corresponds to a three-technical replicate mean, and the error bars indicate standard deviations (for each biomarker and in each time frame, three independent clones were ground together).

Figure 4: Impact of various Cd concentrations on Myriophyllum alterniflorum biomarkers. Five concentrations of CdCl$_2$ were used and supplemented (0.5, 1, 4, 7, 10 µg/L) in vitro in the synthetic oligotrophic medium. The kinetic responses of three biomarkers were analyzed over
27 days: G6PDH, MDA, and vitamin E. Each histogram bar corresponds to a three-technical replicate mean, and the error bars indicate standard deviations (for each biomarker and in each time frame, three independent clones were ground together).

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

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

Figure 7: Graphical representation of the correlation between *Myriophyllum alterniflorum* vitamin E content and Cu concentration measured in water at 14 different *in situ* sites after: 1 day (A), 3 days (B), 6 days (C), 8 days (D), 14 days (E), 21 days (F), 28 days (G), and 35 days (H). (I) corresponds to the compilation of all 5-week data obtained across all sites. On each graph, the $\tau$ Kendall's correlation value and corresponding p-value are indicated. All data
correspond to triplicate, but only the mean without standard deviation is shown in order to avoid overloading the figure.
Watermilfoil is evaluated through biomarkers for its use in water quality monitoring.

- G6PDH, MDA and VitE biomarkers are not correlated with \textit{in vitro} Cu exposures.
- Only MDA and VitE biomarkers are correlated to Cd concentration \textit{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.