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**Are *Myriophyllum alterniflorum* biomarker responses to arsenic stress differentially
affected by hydrodynamic conditions?**

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Abstract

Arsenic (As) is a significant contaminant in the environment and its detection through macrophytes can provide a powerful tool. *Myriophyllum alterniflorum* constitutes a good candidate by virtue of its ability to accumulate contaminants, and moreover its biomarkers can respond to the presence of trace metals and metalloids. The objective of this study therefore is to evaluate the watermilfoil response to As exposure under several hydrodynamic conditions since it is well known that hydrodynamics affect plant functioning. For this purpose, fresh watermilfoil plants are subjected to three hydrodynamic conditions, namely laminar, turbulent and calm, in a synthetic medium either enriched or not by 100 $\mu\text{g.L}^{-1}$ arsenic for 21 days. Growth, pigment content (chlorophyll a, b and carotenoids), respiratory and photosynthetic activities, osmotic potential and hydrogen peroxide concentration are all monitored. Arsenic accumulation is measured separately in the roots and shoots of *Myriophyllum alterniflorum*. On the one hand, it should be noted that arsenic induces: (i) a significant increase in H_2O_2 content; (ii) a decrease in osmotic potential, pigment content, photosynthesis and respiration rates, shoot and root growth; and (iii) an inhibition of shoot branching. Moreover, a higher accumulation of this metalloid in roots than in shoots, regardless of the hydrodynamic condition, is witnessed. While on the other hand, hydrodynamic conditions only affect watermilfoil morphology and arsenic accumulation. Also, the younger and older parts have experienced differential toxic effects. Overall, our results suggest the effective use of *M. alterniflorum* in both water quality biomonitoring and phytoremediation studies.

Keywords: Alternate watermilfoil, biological and physiological endpoints, arsenic, hydrodynamic conditions, oxidative stress.

Abbreviations:

LQ: limit of quantification

MTES: France's Ministry for the Ecological and Solidarity Transition

FW: fresh weight

DW: dry weight

SL: mean total main shoot length

RamifL: mean total length of shoot ramifications

RL: mean total root length.

1- Introduction

Arsenic (As), a metalloid with atomic number 33 and a molar mass of 74.92 g.mol^{-1} , constitutes nearly 0.00021% of the natural elements of the earth's crust. Its critical concentration in streams is $4.2 \text{ }\mu\text{g.L}^{-1}$ (MTES and French Water Agencies, 2003). Arsenic has natural sources like volcanism, wildfires, and the weathering of bedrock regions that contributes to dispersing primary arsenic minerals like arsenopyrite (FeAsS), realgar (AsS) and orpiment (As_2S_3), known to form a geochemical background. The main sources of arsenic are industrial and agricultural uses, including (gold) mining and smelting activities, the combustion of municipal solid wastes and coal fly ash, pharmaceutical wastes, and the use of arsenic pesticides (Laperche *et al.*, 2003; Punshon *et al.*, 2017). Four degrees of oxidation have been identified for As, i.e.: -III, 0, +III, and +V. The toxicity of As compounds decreases as follows: $\text{AsH}_3 \text{ gas} > \text{As (-III)} > \text{As (III)} > \text{As (V)} > \text{methylated compounds}$ (Sharma and Sohn, 2009). In addition, arsenic can be

distinguished in: (i) organic forms, which are mainly arsenobetaine, arsenocholine and mono- and di-methyl derivatives; and (ii) inorganic compounds constituted primarily of arsenite (AsIII) and arsenate (AsV) (Farooq *et al.*, 2016; Singh *et al.*, 2015; Tangahu *et al.*, 2011). The speciation and mobility of arsenic in water are related to both pH and oxido-reduction potential. In natural waters, the main forms are arsenite under reducing conditions in groundwater and arsenate in surface waters (Cullen and Reimer, 1989; Villaescusa and Bollinger, 2008).

Arsenic as a non-essential element for plant growth has no specific and selective transporters (Li *et al.*, 2016). Due to the arsenate/phosphate analogy, arsenate uptake in plant cells occurs via the phosphate transport system (Bakhat *et al.*, 2017; Farooq *et al.*, 2016). In aqueous solutions, arsenate, an oxyanion, actually exists in three forms, with corresponding pKa values (2.2, 7.0 and 11.5) similar to phosphate pKa (2.1, 7.2 and 12.7) (Elias *et al.*, 2012; Tawfik and Viola, 2011). In plants, arsenic is preferentially accumulated in roots (Mazej and Germ, 2009; Vromman *et al.*, 2011; Yabanli *et al.*, 2014). It is well known that As reduces photosynthesis and growth, destroys the chloroplast membrane, reduces chlorophyll synthesis, produces reactive oxygen species (ROS) in leading to deleterious effects in cells (membranes, DNA, photosynthetic pigments) and lastly decreases osmotic potential (Cao *et al.*, 2004; da-Silva *et al.*, 2017; Li *et al.*, 2011; Meharg and Macnair, 1992; Mkandawire and Dudel, 2005; Patra *et al.*, 2004; Rahman *et al.*, 2007b; Robinson *et al.*, 2006; Stoeva *et al.*, 2005; Stoeva and Bineva, 2003; Krayem *et al.*, 2017).

The bioindication of water pollution has been using aquatic macrophytes for several decades (Lagadic *et al.*, 1998), and this process became incorporated into the European Water Framework Directive (Aguiar *et al.*, 2014; Delmail, 2014). Aquatic macrophytes may be impacted not only by the toxic effects of xenobiotics (Pollard *et al.*, 2014) but also by other biotic and abiotic factors, such as hydrodynamic conditions (Atapaththu *et al.*, 2015; Atapaththu

and Asaeda, 2015) or nutrient enrichment (Krayem *et al.*, 2016a, 2016b). It has been demonstrated that water movement plays a key role since it regulates freshwater macrophyte metabolism and growth (Madsen *et al.*, 2001). Furthermore, mechanical stresses leading to hormonal imbalance, ROS generation or lipoperoxidation can be generated by water movement (Ellawala *et al.*, 2013, 2011). To reinforce the use of macrophyte biomarkers in environmental conditions, the interactions between aquatic plants and these environmental factors must be elucidated.

Myriophyllum alterniflorum, also known as alternate watermilfoil, is a submerged aquatic macrophyte found in European inland oligotrophic freshwaters. As demonstrated previously, watermilfoil can accumulate metal or metalloid like copper, cadmium and arsenic (Decou *et al.*, 2019, 2018; Delmail *et al.*, 2011a, 2011b; Krayem *et al.*, 2016b, 2018); until now however, the effect of hydrodynamics on the physiology of submerged macrophytes has rarely been evaluated (Atapaththu *et al.*, 2015; Atapaththu and Asaeda, 2015). In watermilfoil, arsenic could induce a stress leading to a change in biochemical, physiological and morphological biomarkers. In addition, arsenic stress may be modulated by hydrodynamic conditions affecting this macrophyte in the stream.

The aim of this study therefore is to evaluate the combined effect of arsenic stress and hydrodynamics on the response of watermilfoil biomarkers. Moreover, the influence of hydrodynamic conditions on arsenic accumulation is determined and the effects of this accumulation in *Myriophyllum alterniflorum* roots and shoots are investigated.

2. Materials and methods

2.1 Plant material, growth conditions and acclimation

The *M. alterniflorum* strain was previously established by Delmail *et al.* (2011a). After 30 days of proliferation in Murashige and Skoog medium (Murashige and Skoog, 1962), the plant clones were acclimatized for 21 days in Vienne oligotrophic synthetic medium (Krayem *et al.*, 2016a, 2016b, 2018) (the Vienne oligotrophic medium composition is given in the Supplementary Data). Nitrate, ammonium and phosphate concentrations were fixed according to the system of surface water quality evaluation (MTES and French Water Agencies, 2003) at 2 mg.L⁻¹ (32 µM), 0.1 mg.L⁻¹ (5.5 µM) and 0.1 mg.L⁻¹ (1.05 µM), respectively. Plant acclimation was performed in a culture box (Steri Vent High Model 80 × 100 × 100 mm, KALYS SA) containing 400 mL of sterilized medium (20 min, 121°C in an autoclave (DB-90, Systec)) and maintained in a growth cabinet set at 26° ± 2°C, with a 16-h photoperiod and a light intensity of 13.1 ± 0.6 W.cm⁻² (Supra'Lux, Actizoo, 30 W).

2.2 Experimental set-up

In order to simulate environmental conditions, an aquarium system was created with water recirculation (see Fig. 1 in the Supplementary Data). Each aquarium (L=120 cm; W=40 cm, H=49 cm) was filled with 150 L of Vienne oligotrophic synthetic medium (see composition in the Supplementary Data). The treated aquarium was supplemented with 100 µg.L⁻¹ of As (V) Na₂HAsO₄ · 7H₂O (Normapur, Prolabo 99.9%), while another aquarium with no added arsenic served as the control. A channel (pipe half-sectioned in the lengthwise direction, L = 135 cm; Ø = 16.5 cm) was installed over the aquarium. Inside the aquarium, a pump (Laguna power jet, 7600 L/h, 75 W) transferred water from the aquarium to the top of the channel, allowing water to cascade into the aquarium at the bottom of the channel. Three distinct hydrodynamic zones were thus created in this system, namely: calm (in the aquarium), turbulent (at the waterfall from the channel into the aquarium), and laminar (in the channel with a water velocity of approx. 3 cm.s⁻¹

¹). In each zone, 35 grams of fresh watermilfoil were exposed. The younger and older parts of watermilfoils shoots were distinguished. For a morphological biomarker analysis, six node explants (six replicates) were affixed onto a Plexiglas square (10 cm x 10 cm) in two hydrodynamic zones, an intermediate zone and a laminar zone, for 19 days. Three independent experiments were conducted and for each experiment, at least three replicates were analyzed. (For more details about the experimental set-up, see Supplementary Data.)

2.3 Arsenic monitoring

The As concentrations in plants (roots and shoots) were determined by means of ICP-MS (Agilent Technology, ICP-MS 7700). SLRS-5 (river water reference for trace metals, density = 1.0007 g.mL⁻¹) was used as the certified reference for purposes of analysis, with a limit of quantification (LQ) = 0.05 µg As.L⁻¹. 500 mg of fresh watermilfoil were therefore dried at 60°C for 48 h and then digested in 4 mL of a mixture of HNO₃ (Xilab for trace element analysis, 69%) and H₂O₂ (Prolabo, 30%) (50/50 v/v) at 150°C for 2 h, adjusted afterwards to 10 mL with MilliQ-Water and filtered with a 0.2-µm syringe filter (0.2 µm, Whatman). The Bioconcentration Factor (BCF) was calculated by applying Equation 1:

$$\text{BCF (L.kg}^{-1}\text{)} = [\text{M}] \text{ plants} / [\text{M}] \text{ water (Eq. 1)}$$

where [M] plants is defined as the metal concentration in plants (shoots or roots), in µg.kg⁻¹ FW, and [M] water the metal concentration in water, in µg.L⁻¹. All these calculations were performed on three replicates of plants and water samples in each of the three independent experiments.

2.4 Biomarker analysis

2.4.1 Biochemical biomarkers

2.4.1.1 Hydrogen peroxide content (H₂O₂)

The H₂O₂ content was measured according to Zhou *et al.* (2006); details are provided in the Supplementary Data.

2.4.1.2 Chlorophyll a, b and carotenoid contents

Pigment quantification was based on the methods implemented by Delmail *et al.* (2011a), with these experimental conditions also being detailed in the Supplementary Data.

2.4.2 Physiological biomarkers

2.4.2.1 Osmotic potential (Ψ_{scor})

The osmotic potential (Ψ_s) was measured using a vapor pressure osmometer (Micro Osmometer Manual, Löser Messtechnik) and in accordance with the method implemented by Krayem *et al.* (2018). Further details are provided in the Supplementary Data.

2.4.2.2 Photosynthetic and respiration rates

For each independent experiment, both photosynthetic and respiration rates were measured on three replicates at 1 and 21 days in an aquarium system using a Clark-type oxygen electrode (DW 1 Oxygen Electrode Chamber, Hansatech Instruments, Ltd.) and by following the method detailed in the Supplementary Data.

2.4.3 Morphological biomarkers

For each independent experiment, six watermilfoil shoots affixed onto Plexiglas squares were monitored in an aquarium for 19 days. Two squares per aquarium were laid out in the intermediate and laminar zones, as previously described in Section 2.2. For each square, the mean total main shoot length (SL), mean total length of shoot ramifications (RamifL) and mean

total root length (RL) were measured on plant pictures taken by a Sony camera (14 M pixels) every third day, then analyzed with the Visilog Viewer 6.8 software (NOESIS).

2.5 Statistical analysis

For each independent experiment and for each hydrodynamic zone, our results relied on three replicates (N=3), except for the growth parameters, which were based on six replicates (N=6). Data normality was verified by a kernel density estimation, along with typical Lilliefors and Shapiro tests. The effects of As on pigment content, hydrogen peroxide, osmotic potential and morphology at 21 days were assessed by the mixed ANOVA method, followed by a Sidak test to compare differences between control plants and arsenic-treated plants, as well as between younger and older plant parts. Arsenic accumulation in the various plant parts and the bioconcentration factor (BCF) were both tested by means of the Bonferonni test. All tests were performed at a 5% significance level using the Systat 11 software package for Windows.

3. Results and discussion

3.1 As internalization in watermilfoil

Arsenic concentrations ranged between 200 and 500 $\mu\text{g.g}^{-1}$ plant dry weight (DW) in this experiment, while they only reached 150 $\mu\text{g.g}^{-1}$ DW in a previous box experiment (Krayem *et al.*, 2016a) conducted at 21 days of exposure for each system (Table 1). This higher arsenic accumulation in watermilfoil from the aquarium system was mainly due to the higher level of exposure to arsenic. If the arsenic concentration was indeed identical (100 $\mu\text{g.L}^{-1}$) in the two systems (150-L aquarium and 400-mL boxes), then the biomass/As ratio in the aquarium was 89 times higher than in the boxes.

In addition, hydrodynamics affected the differential accumulation of arsenic by roots and shoots. A 57%, 57% and 44% higher As accumulation in roots than in shoots was recorded (p-value =

0.001) under calm, turbulent and laminar conditions, respectively. These results were also assessed by calculating the BCFs in roots and shoots within each of the three hydrodynamic zones. A 58% and 51% higher BCF (see Table 1) in roots than in shoots was recorded in the calm and turbulent zones, respectively. Similarly, in the laminar zone, a 45% increase in the BCF in roots, compared to shoots, was found. It appears that in roots, As accumulation was highest in the calm zone. An increase in the arsenic content of roots located in the calm zone, by about 8% and 23%, was noted compared to the turbulent and laminar zones, respectively. It could be hypothesized that these differences in As accumulation are correlated with water flow velocity. It could indeed be assumed that changes in water velocity affect plant metabolism and growth, as has been reported by many authors (for a comprehensive review, see Bornette and Puijalon, 2011; Franklin *et al.*, 2008). For example, Strand and Weisner (2001) indicated that *M. spicatum* at sheltered sites increase (or at least do not decrease) their below-ground biomass. This greater allocation to below-ground organs could influence absorption and explain the increase in arsenic content observed in our experiment. Moreover, as reported by Franklin *et al.* (2008), photosynthetic and nutrient uptake rates are positively correlated with low water velocity (0 - 0.1 m.s⁻¹). Accordingly, our results on *M. alterniflorum* should be explored by further experiments since the influence of flow velocity on aquatic macrophytes is a very complex process. It is well documented that at moderate water flow rates as velocity increases, the diffusive boundary layer around the plant surface erodes more substantially, thus leading to enhanced nutrient and gas fluxes and hence photosynthesis and growth. In contrast, Franklin *et al.* (2008) indicated that *M. spicatum* belong to the species most susceptible to increased velocity due to high hydraulic resistance and low anchoring strength. Increasing the velocity actually leads to physical and

mechanical stresses due to greater drag forces, which in turn can lead to stem breakage and uprooting.

In conclusion, a higher flow velocity induces the lowest As accumulation in roots, while in shoots, the As level was practically similar across the three hydrodynamic zones. The BCF was higher in roots than in shoots for all three conditions and higher in the calm zone, whereas the shoot/root ratio was lower in the laminar zone than in the two other zones (1.77 vs. 2.29). Arsenic is a non-essential element for plants, and it is well known that the root endodermis constitutes a barrier to the root-to-shoot translocation of toxic contaminants (Mazhoudi *et al.*, 1997). In addition, several studies have pointed to roots as the preferential site of arsenic accumulation in plants (Mazej and Germ, 2009; Vromman *et al.*, 2011; Yabanli *et al.*, 2014). A BCF of 4,000 L.kg⁻¹ (based on dry matter) was observed in the roots of *Hydrilla verticillata* after exposure to 0.15 µg.L⁻¹ of arsenic for 4 days (Xue and Yan, 2011). Mazej and Germ (2009) found a BCF of 1.5 L.kg⁻¹ in the roots, compared with a BCF of 0.045 L.kg⁻¹ in the shoots, of *Najas marina* after a treatment with 10.6 mg/kg of arsenic in the sediment. This reduced root-to-shoot transfer of arsenic may be due to phytochelatin-mediated As vacuolar sequestration in root cells (Gupta *et al.*, 2004; Hasanuzzaman *et al.*, 2015; Zhao *et al.*, 2009).

3.2 Biomarker analysis

3.2.1 Biochemical biomarkers

3.2.1.1 H₂O₂ content

H₂O₂ content was not affected by the hydrodynamic conditions in control plants (Fig. 1). When comparing young and old parts, as opposed to As-treated plants, the control plants did not display any significant difference (p-value = 0.154). At 21 days, in the As-treated plants, a

significant increase in H₂O₂ content of 96%, 106% and 100% (p-value = 0.001) in the young parts and 85%, 140% and 83% (p-value = 0.004) in the old parts of As-exposed plants, compared to the control specimens, were observed for the calm, turbulent and laminar zones, respectively (Fig. 1). Arsenic toxicity appears on the tissues of both young and old parts, generating an oxidative stress characterized by the formation of hydrogen peroxide (Hasanuzzaman *et al.*, 2015; Mallick *et al.*, 2011).

In contrast, at 21 days in As-treated plants, a significant increase in H₂O₂ content of 28%, 49% and 29% (p-value = 0.049) is observed in the older parts for the calm, turbulent and laminar zones, respectively (Fig. 1), as compared to the younger parts. Similarly, a higher As accumulation in older than younger fronds of the terrestrial plant *Pteris vittata* could be observed (7.575 µgAs.g⁻¹ DW / 4.893 µgAs.g⁻¹ DW) after 12 weeks of cultivation in 97 µgAs.g⁻¹ soil. This differential accumulation appears to produce an As detoxification mechanism as older senescent leaves fall (Zhang *et al.*, 2002). Bouazizi *et al.* (2010) discovered that, unlike in older leaves, hydrogen peroxide content remained stable in the young leaves of *Phaseolus vulgaris* watered with 3.2 mg.L⁻¹ Cu, thus suggesting a greater stimulation of peroxidase activity in older rather than younger tissues. Along the same lines, our results have suggested a higher oxidative stress in older shoots. This differential effect of As on older parts could be explained by the increased activity of the enzymatic antioxidant system (superoxide dismutase, ascorbate peroxidase, peroxidase) in younger parts, contributing to ROS limitation in order to protect young tissues from ROS degradation, i.e. a phenomenon previously observed in several species in response to various trace elements (da-Silva *et al.*, 2017; Hasanuzzaman *et al.*, 2015; Delmail and Labrousse, 2012). Another aspect could be the preferential As sequestration in old leaves to protect the younger ones.

3.2.1.2 Pigment content

Pigment levels were not affected by the hydrodynamic conditions in control plants (Fig. 2). In As-treated plants, a significant decrease in pigment content, equal to 22%, 29% and 22% for Chl a (p-value = 0.026) (Fig. 2) and 31%, 23%, and 10% for Chl b (p-value = 0.048), was observed (Fig. 2) in the younger parts for the calm, turbulent and laminar zones, respectively. This reduction reached 22%, 24% and 18% for Chl a (p-value = 0.026) (Fig. 2) in the older parts compared to the control for the calm, turbulent and laminar zones, respectively. On the other hand, chlorophyll b in the older parts of the As-treated plants did not change significantly (p-value = 0.091) over time compared to the controls for the three hydrodynamic zones, respectively (Fig. 2). In contrast, the carotenoid concentration (Fig. 2) did not differ significantly in the young shoots vs. the control (p-value = 0.387), whereas in old shoots a significant increase of approximately 25% (p-value = 0.014) was observed for the calm, turbulent and laminar zones. Arsenic therefore caused a decrease of Chl a and b, while the carotenoids only increased in the older parts. Duman *et al.* (2010) found in *Lemna minor* a decrease in chlorophyll pigment content during arsenic stress (0.079-5.056 mg.L⁻¹ As (V) in 400-mL Hoagland medium over 6 days). Similarly, Ozturk *et al.* (2010) demonstrated this same feature in *Nasturtium officinale* (after exposing 4 g of plants to doses of 0.79 mg.L⁻¹ to 3.9 mg.L⁻¹ of As(III) in 400 mL of Hoagland medium for 7 days). Srivastava *et al.* (2013) indicated that in *Hydrilla verticillata*, exposure to 37.5 mg.L⁻¹ As for 96 hours induced a 24% decrease in chlorophyll content. The increase in carotenoid content observed in this study may however reflect one of the non-enzymatic defense mechanisms adopted by the plant to protect its chlorophyll pigments from oxidative degradation (Ferrat *et al.*, 2003). In contrast, a 2-mg.L⁻¹ arsenate exposure on two aquatic species, *Vallisneria gigantea* and *Azolla filliculoides*, induced an increase of fluorescence

in the range of 400 to 500 nm; this finding was correlated with an increase in the biosynthesis of carotenoids and flavonoids, i.e. two compounds occurring in the arsenic defense system (Iriel *et al.*, 2015).

When comparing older and younger parts of watermilfoil under each hydrodynamic condition, a decrease of 26%, 38% and 38% (p-value = 0.002) for Chl a and 45%, 39%, and 46% (p-value = 0.009) for Chl b were observed in the older parts relative to the younger parts of the control plants in the calm, turbulent and laminar zones, respectively (Fig. 2). Similarly, in As-treated plants, a reduction of 26%, 33% and 35% for Chl a (p-value = 0.005) (Fig. 2) and 37%, 47% and 46% (p-value = 0.003) for Chl b (Fig. 2) had also been recorded. Alternatively, the carotenoid contents remained stable in both the control specimens (p-value = 0.959) and As-treated plants (p-value = 0.086) regardless of the hydrodynamic condition. Therefore, the As effect on older parts was greater than on younger ones, especially for chlorophylls a and b. The decrease in photosynthetic pigment contents could be due to: (i) lipid peroxidation by ROS and chloroplast membrane degradation (Upadhyay and Panda, 2009), (ii) chlorophyll degradation by chlorophyllase (Sharma and Dubey, 2005), or (iii) acceleration of a tissue aging process called "senescence" resulting in a decrease in pigment content due to resource remobilization (Delmail and Labrousse, 2012). The stability of the carotenoid contents in the younger parts and their increase in the older parts is reflected by the major role these contents play in defending and protecting the other pigments from oxidative degradation (Ferrat *et al.*, 2003).

3.2.2 Physiological biomarkers

3.2.2.1 Osmotic potential

The hydrodynamic conditions did not affect the osmotic potential of control plants (p-value = 0.316). In addition, no significant difference was observed between younger and older parts in the control specimens (p-value = 0.416) (Fig. 3).

However, in As-treated plants (Fig. 3), significant decreases in osmotic potential of 37%, 19% and 22% in the younger parts (p-value = 0.002) and 26%, 24% and 26% in the older parts (p-value = 0.002) were observed for the calm, turbulent and laminar zones, respectively (Fig. 3).

The exposure of plants to arsenic can in fact cause an increase in the protein, soluble sugar, proline and glycine-betaine contents in vacuoles (Hasanuzzaman *et al.*, 2015). This accumulation leads to an increase in solute concentrations and, consequently, contributes to decreased osmotic potential (Garg and Singla, 2011; Hasanuzzaman *et al.*, 2015; Stoeva *et al.*, 2005; Yabanli *et al.*, 2014). Tripathi *et al.* (2014) studied the effect of increasing concentrations of As (V) from 0 to 18.5 mg.L⁻¹ on the macrophyte *Najas indica* by measuring the amino acid concentration. They found that the total concentration of amino acids and proteins increased by 54% and 42% respectively after exposure to 18.5 mg.L⁻¹ of As over 2 days. This increase in the concentration of amino acids and proteins in plant cells could induce a decrease in osmotic potential and moreover explains the result obtained in this study. In addition, Srivastava *et al.* (2013) showed that *Hydrilla verticillata* reduced its water use and increased its transpiration during As exposure (37.5 mg.L⁻¹ and after 96 hours). This reduction led to an increased solute concentration and hence a decrease in osmotic potential. Also, this result is similar to the observation by Delmail *et al.* (2011a) highlighting a 27% and 65% decrease in the osmotic potential of younger and older leaves of *Myriophyllum alterniflorum* during a 30-day exposure to 100 µg Cu.L⁻¹. Moreover, Krayem *et al.* (2018) reported a decrease in the osmotic potential of 22%, 16% and 20% in younger parts (p-value = 0.008) and of 22%, 18%, and 17% in older parts (p-value = 0.008)

compared to the control specimens in the calm, turbulent and laminar zones, respectively, in *Myriophyllum alterniflorum* plants after exposure to 100 $\mu\text{g.Cu.L}^{-1}$.

No significant difference between young and old parts was found for either the control group (p-value = 0.416) or the As-treated plants (p-value = 0.420). In addition, there was no significant difference in osmotic potential across the three zones and between the control and As-treated plants (p-value = 0.388). Under our experimental conditions therefore, the effect of arsenic did not vary between the older and younger parts of watermilfoil. This result differs from the 38% decrease in osmotic potential observed in older leaves of *Myriophyllum alterniflorum*, compared to younger leaves, at 30 days of exposure to 100 $\mu\text{g Cu.L}^{-1}$, as reported by Delmail *et al.* (2011a), whose medium differed from ours, particularly in nitrates and phosphates, which could explain this discrepancy.

3.2.2.2 Photosynthetic and respiratory activities

No significant effect of hydrodynamic conditions on respiration and net photosynthesis (NP) in the control plants was observed (p-values = 0.138 and 0.855, respectively) (Fig. 4). In the As-treated plants however, a significant decrease in respiration, ranging from 30% to 50% (p-value = 0.001), and in photosynthesis, ranging from 30% to 40% (p-value = 0.001), was recorded on Day 1 and Day 21, respectively, within the three zones (Fig. 4).

The As toxicity clearly induced a response of watermilfoil biochemical and physiological biomarkers. Exposure to 143 $\mu\text{g As.g}^{-1}$ FW led to a decrease in respiration and NP. The oxidative stress generated by arsenic can indeed destroy thylakoid membranes, thus reducing pigment contents and affecting the photosystem II (Prasad *et al.*, 2001). Stoeva and Bineva (2003) showed that arsenic can induce chloroplast membrane destruction, leading to decreased photosynthesis. In *Hydrilla verticillata*, a 32 mg.L^{-1} As exposure for 96 h induced a decrease in

net photosynthesis and in Chl a, Chl b and Car contents (Srivastava *et al.*, 2013). This reduction could be due to: (i) chlorophyll degradation by ROS, (ii) a reduction in photosystem II efficiency, and/or (iii) a dysfunction of the thylakoid electron transport chain (Duman *et al.*, 2010; Hasanuzzaman *et al.*, 2015; Rahman *et al.*, 2007a). Furthermore, arsenic and especially As (V) can form a complex with adenosine diphosphate by means of phosphate substitution. ADP-As (V) inhibits the synthesis of the adenosine triphosphate (ATP) necessary for respiration in the mitochondrial electron transport chain as well as for photosynthesis in the thylakoid electron transport chain. Such a mechanism leads to a reduction of respiratory and photosynthetic activities in plant cells (Elias *et al.*, 2012; Finnegan and Chen, 2012).

3.2.3 Morphological biomarkers

In As-treated plants, a significant decrease in shoot length (SL) (Fig. 5a) by 15% and 16% (p-value = 0.0260) at 19 days was observed for the intermediate and laminar zones, respectively, compared to the control plants. Similarly, RL (Fig. 5b) decreased by 35% and 52% (p-value = 0.005) at T = 19 days of As exposure for the intermediate and laminar zones, respectively, relative to the control plants. This significant reduction in stem and root lengths reflects As toxicity (Hasanuzzaman *et al.*, 2015). Gusman *et al.* (2013) observed a 38% decrease in *Lactuca sativa* leaf length and a 50% decrease in root length after exposure to 3.4 mg.L⁻¹ As. Liu *et al.* (2005) found a reduction in the length of the main stem and root of 3.9% and 8.6%, respectively, after treating *Triticum aestivum* with 0.5 mg.L⁻¹ As. In *Pisum sativum* after 24 h of exposure to 0.2 mg.L⁻¹ As (V), Dho *et al.* (2010) observed that the root growth and mitotic index decreased by 42% and 20%, respectively. This reduction in meristematic activity was correlated with ROS production affecting root cells mitosis. In addition, in *Oryza sativa* exposed to 7.8 and 15.6 mg.L⁻¹ As for 24 h, Singh *et al.* (2009) recorded a 23% and 37% decrease in root length; these

authors also noted a 81% and 30% increase in peroxy radical $O_2^{\cdot-}$ and hydrogen peroxide at 15.6 mg.L⁻¹ As. As previously observed in the literature, oxidative stress due to As could thus occur in roots, decreasing root length after an exposure to 100 µg As (V).L⁻¹.

No shoot branching was observed in control and As-treated plants in both hydrodynamic zones.

In addition, a significant decrease in SL (p-value = 0.05) and RL (p-value = 0.001) was noted in plants from the intermediate zone as compared to the laminar zone (Figs. 5a, b). As previously pointed out, flow velocity exerts a significant effect on macrophyte growth, from a stimulating effect at low and moderate velocities to an inhibiting effect at higher velocities; moreover, water movement could generate physical and mechanical stresses, in inducing morphological changes like dwarfed growth form (Bornette and Puijalon, 2011; Franklin *et al.*, 2008; Madsen *et al.*, 2001). It should be noted that water turbulence can affect growth more drastically than laminar flow, as demonstrated in *Elodea nuttallii* by Atapaththu and Asaeda (2015). Water turbulence can induce chlorosis, plasmolysis in leaf cells, and the accumulation of organic acids (like citrate, oxalate and ascorbate) and amino acids, which could interfere with cell metabolism and inhibit plant growth (Atapaththu *et al.*, 2015; Atapaththu and Asaeda, 2015). Lastly, these authors also reported ultrastructural changes, such as starch granule accumulation in chloroplasts and cell wall thickening linked to cellulose and lignin accumulation.

Conclusion

In our study, hydrodynamics did not influence the response of the watermilfoil biomarkers tested, except for the growth parameters; this response of several aquatic macrophytes species to water movement is well known since water velocities and turbulence induce mechanical stress

and lead to a decline in plant growth. Hydrodynamics did however affect arsenic accumulation and acted differentially on the roots and shoots. The arsenic concentration observed in roots was higher than that in shoots, most likely resulting from a mixed response of water velocity inducing a below-ground biomass accumulation along with stress-inducing As sequestration in the vacuoles of root cells. In this manner, the watermilfoil protected its shoots from As toxicity, and particularly the younger parts since As accumulation and toxicity biomarker responses are higher in the older parts.

Further studies are needed to determine the exact contribution of flow velocity, water turbulence and trace element stress on aquatic macrophyte biomarkers.

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Figure 1: Effect of arsenic supply on hydrogen peroxide (H_2O_2) content (μM based on FW) in shoots of watermilfoil plants - Controls and Treated with $100 \mu\text{g.L}^{-1}$ As in a recirculated system at 21 days. The values shown are means $\pm$ standard error of three independent experiments ($N=9$). The uppercase letters compare the H_2O_2 concentrations at the same plant parts (old or young) across the hydrodynamic zones, while lowercase letters compare the H_2O_2 concentrations within the same hydrodynamic zone between the different plant parts (old vs. young) on the Control and As-treated plants. The means followed by the same letter do not differ in the Sidak test at a 5% significance level.

Figure 2: Effect of arsenic supply on pigment contents (in $\mu\text{g.g}^{-1}$ FW) in shoots of watermilfoil plants - Controls and Treated with $100 \mu\text{g.L}^{-1}$ As in a recirculated system at 21 days. The values shown are means $\pm$ standard error of three independent experiments ($N=9$). The uppercase letters compare the pigment concentrations at the same plant parts (old or young) across the hydrodynamic zones, while lowercase letters compare the pigment concentrations within the same hydrodynamic zone between the different plant parts (old vs. young) on the Control and As-treated plants. The means followed by the same letter do not differ in the Sidak test at a 5% significance level. (FW: Fresh Weight, Chl a: chlorophyll a, Chl b: chlorophyll b, car: carotenoid)

Figure 3: Effect of arsenic supply on the osmotic potential (Ψ_{scor}) $\mu\text{osmol.kg}^{-1} \text{H}_2\text{O}$) in shoots of watermilfoil plants - Controls and Treated with $100 \mu\text{g.L}^{-1}$ As in a recirculated system at 21 days. The values shown are means $\pm$ standard error of three independent experiments ($N=9$). The uppercase letters compare the osmotic potential at the same plant parts (old or young) across the

various hydrodynamic zones, while lowercase letters compare the osmotic potential within the same hydrodynamic zone between the different plant parts (old vs. young) on the Control and As-treated plants. The means followed by the same letter do not differ in the Sidak test at a 5% significance level.

Figure 4: Effect of arsenic supply on the net photosynthesis and respiration in the watermilfoil plants - Controls and Treated with $100 \mu\text{g.L}^{-1}$ As in a recirculated system. The values shown are means $\pm$ standard error of three independent experiments (N=9), expressed in $\text{mmolO}_2.\text{mL}^{-1}.\text{s}^{-1}.\text{g}^{-1}$ FW. Net photosynthesis (NP), Respiration (Res). The uppercase letters compare the respiratory and photosynthetic activities in the control and As-treated plants separately across hydrodynamic zones, while lowercase letters compare the respiratory and photosynthetic activities within the same hydrodynamic zone between the Control and As-treated plants. The means followed by the same letter do not differ in the Sidak test at a 5% significance level.

Figure 5: Effect of arsenic supply on the mean total main stem length (SL) (A) and mean total root length (RL) (B) of watermilfoil plants - Controls and Treated with $100 \mu\text{g.L}^{-1}$ As in a recirculated system for 19 days. The values shown are means $\pm$ standard error of three independent experiments (N=18), expressed in cm. The uppercase letters compare the SL and RL in the control and As-treated plants separately across hydrodynamic zones, while lowercase letters compare the SL and RL within the same hydrodynamic zone between the Control and As-treated plants. The means followed by the same letter do not differ in the Sidak test at a 5% significance level. Inter: intermediate, Lam: Laminar.

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706 **List of tables:**

707 **Table 1:** Arsenic accumulation (in $\mu\text{g.g}^{-1}$ DW) and bioconcentration factor (BCF, in L.kg^{-1}) in
708 the shoots and roots of watermilfoil plants - Controls and Treated with $100 \mu\text{g.L}^{-1}$ As in a
709 recirculated system by considering three hydrodynamic conditions (calm, turbulent and laminar)
710 at 21 days. The values shown are means $\pm$ standard error of three independent experiments
711 (N=6). The uppercase letters compare the arsenic concentration for the same plant parts across
712 the various hydrodynamic zones, while lowercase letters compare the arsenic concentration
713 within the same hydrodynamic zone across the various plant parts. The means followed by the
714 same letter do not differ in the Bonferroni test at a 5% significance level.

A

B

	Plant parts	Hydrodynamic conditions		
		Calm zone	Turbulent zone	Laminar zone
[As]	Stem+ leaves	218±1 ^{Aa}	200±1 ^{Bc}	218±2 ^{Ae}
μg.g ⁻¹ DW	Root	500±2 ^{Ab}	459±1 ^{Bd}	387±1 ^{Cf}
BCF (L.kg ⁻¹)	Stem+ leaves	176±40 ^{Aa}	172±60 ^{Aa}	169±70 ^{Aa}
	Root	414±80 ^{Ab}	346±50 ^{Bb}	308±44 ^{Cb}