Effects of cadmium, inorganic mercury and methyl-mercury on the physiology and metabolomic profiles of shoots of the macrophyte Elodea nuttallii

Claudia Cosio, D Renault

To cite this version:
Claudia Cosio, D Renault. Effects of cadmium, inorganic mercury and methyl-mercury on the physiology and metabolomic profiles of shoots of the macrophyte Elodea nuttallii. Environmental Pollution, 2020, 257, pp.113557. 10.1016/j.envpol.2019.113557. hal-02393954

HAL Id: hal-02393954
https://univ-rennes.hal.science/hal-02393954
Submitted on 3 Feb 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.
Effects of cadmium, inorganic mercury and methyl-mercury on the physiology and metabolomic profiles of shoots of the macrophyte *Elodea nuttallii*

Claudia Cosio*1,2* and David Renault2,3

1 Université de Reims Champagne Ardennes, UMR I 02 INERIS-URCA-ULH SEBIO, F-51687 Reims, France
2 Université de Rennes 1, UMR 6553 EcoBio CNRS, F-35042 Rennes, France
3 Institut Universitaire de France, 1 rue Descartes, 75231 Paris CEDEX 05, France

*Corresponding author: claudia.cosio@univ-reims.fr
ABSTRACT
Macrophytes are known to bioaccumulate metals, but a thorough understanding of tolerance strategies and molecular impact of metals in aquatic plants is still lacking. The present study aimed to compare Hg and Cd effects in a representative macrophyte, *Elodea nuttallii* using physiological endpoints and metabolite profiles in shoots and cytosol. Exposure 24h to methyl-Hg (23 ng·L⁻¹), inorganic Hg (70 ng·L⁻¹) and Cd (281 µg·L⁻¹) did not affect photosynthesis, or antioxidant enzymes despite the significant accumulation of metals, confirming a sublethal stress level. In shoots, Cd resulted in a higher level of regulation of metabolites than MeHg, while MeHg resulted in the largest number of regulated metabolites and IHg treatment regulated no metabolites significantly. In cytosol, Cd regulated more metabolites than IHg and only arginine, histidine and mannose were reduced by MeHg exposure. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of data suggested that exposure to MeHg resulted in biochemical changes including aminoacyl-tRNA biosynthesis, glycine, serine and threonine metabolism, nitrogen metabolism, arginine and proline metabolism, cyanoamino acid metabolism, while the treatment of Cd stress caused significant variations in aminoacyl-tRNA biosynthesis and branched-chain amino acids pathways. Data supports an impact of MeHg on N homeostasis, while Cd resulted in an osmotic stress-like pattern and IHg had a low impact. Marked differences in the responses to MeHg and IHg exposure were evidenced, supporting different molecular toxicity pathways and main impact of MeHg on non-soluble compartment, while main impact of IHg was on soluble compartment. Metabolomics was used for the first time in this species and proved to be very useful to confirm and complement recent knowledge gained by transcriptomics and proteomics, highlighting the high interest of multi-omics approaches to identify early impact of environmental pollution.

Keywords: amino acids, cytosol, macrophyte, shoots, sugars.

Capsule: Metabolomics reveal different cellular toxicity responses to sublethal Cd, MeHg and IHg treatment in *Elodea nuttallii*
1. INTRODUCTION

Anthropogenic activities result in environmental contamination by mercury (Hg) and cadmium (Cd) (Loizeau et al., 2013; Azimi and Rocher, 2016). As a result, biota is affected by the toxicity of these toxic metals, which can also affect human populations via contaminated food (Croteau et al., 2005; Bravo et al., 2010; Niane et al., 2014). Metals can enter the food chain through primary producers, including macrophytes, which represent a key group within communities of shallow water, in terms of biomass, primary production, food source and shelter for fauna. Importantly, macrophytes are also central to oxygen, pollutants and nutrients biogeochemical cycles (Wetzel, 2001; Chambers et al., 2008; Bonanno and Lo Giudice, 2010; Noges et al., 2010). High levels of Cd, inorganic Hg (IHg) and methyl-Hg (MeHg) can be observed in aquatic ecosystems colonized by plants (Samecka-Cymerman and Kempers, 2003; Castro et al., 2009; Regier et al., 2013b). Therefore, bioaccumulation of metals in macrophytes represents a significant and current environmental concern (Beauvais-Fluck et al., 2016; Beauvais-Fluck et al., 2017).

Due to their ability to accumulate and tolerate high concentrations of metals (Regier et al., 2013b; Cosio et al., 2014), aquatic plants are often seen as good candidates for phytoremediation, biomonitoring and ecotoxicology investigations (Valega et al., 2009; Bonanno and Lo Giudice, 2010; Cosio et al., 2014). However, physiological mechanisms associated with metal accumulation and prevention of their toxicity are still poorly understood in those plants, preventing the full optimization of their use for environmental applications. Increasing the knowledge on the biology and physiology of these plants, and more particularly the impact of metal accumulation on their metabolism is therefore a research priority (Cosio et al., 2014).

*Elodea nuttallii* is a macrophyte widely distributed in temperate regions, and it is well-known for its accumulative capacity of metals (Larras et al., 2013; Regier et al., 2013b). Several recent studies used transcriptomics and proteomics approaches to describe cellular toxicity of Cd and Hg in *E. nuttallii*, with the aim of using this plant for active biomonitoring. Data revealed rapid molecular changes in metal-exposed plants (Larras et al., 2013; Regier et al., 2013a; Regier et al., 2013b; Regier et al., 2016; Beauvais-Fluck et al., 2018a; Beauvais-Fluck et al., 2018b). These studies, spanning a wide range of concentrations, globally suggest toxic metals to impact genes and proteins involved in cell structure (lignin content), energy metabolism (sugar and photosynthesis), amino acid metabolism, and to trigger anti-oxidative stress responses (Larras et al., 2013; Regier et al., 2013a; Regier et al., 2013b; Regier et al., 2016; Beauvais-Fluck et al., 2018b). Besides, several differences were observed between IHg and MeHg responses. At the transcriptome level, MeHg deregulated more genes than IHg at similar intracellular concentrations (Beauvais-Fluck et al., 2018b). At the proteome level, 200 ng·L⁻¹ IHg had a significant and similar effect than 500 µg·L⁻¹ Cd exposure, while 30 ng·L⁻¹ MeHg was...
similar to controls, showing non-significant changes in proteome (Larras et al., 2013). Data thus
suggest different cellular toxicity pathways for IHg and MeHg. However, for both transcriptomic and
proteomic, many regulated contigs or peptides had an unknown function indicating considerable
potential for new discoveries in the biology of toxic metals.

Views obtained from transcriptomics, proteomics and physiological studies depict distinct levels of
regulation that exist in the organism. Transcriptomic informs on the level of expression of genes, but
cellular functions of a particular gene are carried out by its protein. Regulation of proteins in turn
results in alterations in levels of small metabolites involved in biochemical reactions in the functional
organism. Gene regulation might happen to maintain protein levels, whereas changes in the
abundance of proteins can be related to posttranslational modifications of proteins. Eventually,
proteins carry out a myriad of functions within the cell, including the metabolism of small molecules
such as amino acids and sugars, known to have functional roles in plant abiotic stress tolerance and
signaling (Yancey, 2005). Physiological adjustments and degradation of pollutants is thus ultimately
sustained by the metabolic network, whose production of energy, and primary and secondary
metabolites, are central for physiological repair and remodeling of cells. In this context,
metabolomic enables a large physiological representation of the effect of pollutants by incorporating
metabolic fluxes, enzymatic kinetics, and the resulting action of the stressors at organismal level.

Metabolic reconfigurations in plants exposed to pollutants, and more generally to a range of abiotic
stressors, result in the up-regulation and down-regulation of specific metabolic functions to sustain
biological functions (sustainers) and control/modulate metabolite fluxes (modulators) (Topfer et al.,
2014; Tohge et al., 2015).

In this context, metabolomics give the opportunity to confirm or reveal effects of toxicants in a way
that cannot be fully exploited by other molecular approaches (e.g. proteomics and transcriptomics).

2. EXPERIMENTAL SECTION

2.1. Lab-ware and reagents

All laboratory material was soaked in 10% v/v HNO₃ (pro analysis, Merck, Nyon, Switzerland)
followed by two 10% v/v HCl (pro analysis, Merck, Nyon, Switzerland) acid baths for ≥ 1 week,
thoroughly rinsed with ultrapure water (<18.2 MΩ, MilliQ Direct system, Merck Millipore, Darmstadt, Germany) and dried under a laminar flow hood. MeHg (CH₃HgCl) standard solution (1 g·L⁻¹) was obtained from Alfa Aesar (Ward Hill, MA, USA), while IHg (Hg(NO₃)₂) and Cd (Cd(NO₃)₂) standard solutions (1 g·L⁻¹) were obtained from Sigma-Aldrich (Buchs, Switzerland).

2.2. Plant culture and exposure to toxic metals

Shoots of *Elodea nuttallii* (Planch.) St. John were collected in Lake Geneva (Switzerland, N46° 16' 28.7" E6° 10' 15.7"), and a culture was established and maintained in microcosms as previously described (Regier et al., 2013b). Cultures and experiments were conducted in the laboratory under controlled conditions (20 °C, 16/8 h with 5.84 W m⁻² photosynthetically active radiation [PAR], 1000 lux, 20 ± 1 °C). We exposed (or not: control) for 24h in triplicate three 10 cm-long shoots without roots in 1L bechers filled with 1.2 µm filtered Lake Geneva water spiked with 30 ng·L⁻¹ MeHg (nominal concentration 30 ng·L⁻¹, effective concentration 23 ± 7 ng·L⁻¹ MeHg), 70 ng·L⁻¹ IHg (nominal concentration 200 ng·L⁻¹, effective concentration 70 ± 14 ng·L⁻¹ IHg) or 280 µg·L⁻¹ Cd (nominal concentration 500 µg·L⁻¹, effective concentration 281 ± 24 µg·L⁻¹ Cd).

2.3. Hg and Cd uptake

After the 24h-long exposure to toxic metals, shoots were rinsed with 1 mM EDTA for IHg or Cd treatments, and with 1 mM EDTA + 1 mM cysteine for MeHg treatments, to assess intracellular metal concentrations (i.e. not extractable portion) (Larras et al., 2013). For total Hg (THg = IHg + MeHg), lyophilized shoots of *E. nuttallii* were analyzed by AMA-254. For MeHg, shoots were ground, freeze-dried, and extracted by HNO₃ leaching/CH₂Cl₂ extraction. Then, they were analyzed by ethylation onto Tenax traps, followed by GC separation and cold vapor atomic fluorescence spectrometry (GC-CV-AFS), as described in Liu et al. (2012). For Cd, shoots were ground, freeze-dried and analyzed by ICP-MS (HP 4500, Agilent) after mineralization into 4 mL HNO₃ s.p. and 1 mL H₂O₂ s.p.

THg and Cd in cytosol (see cytosol extraction protocol below) were measured by cold vapor atomic fluorescence spectroscopy (CV-AFS, 2500 Tekran) and ICP-MS, respectively. MeHg in cytosol was measured by the hydride generation method with cryogenic trapping, gas chromatography and atomic fluorescence spectrometry (CT-GC-AFS) (Stoichev et al., 2004).

For all metal analysis methods, the analytical quality was assured by analyzing certified reference material, blanks and several samples twice.

2.4. Physiological endpoints
Chlorophyll content was measured by spectrophotometry (Cosio and Dunand, 2010). Chlorophyll fluorescence allows determining PSII efficiency, and was measured using a Handy PEA fluorimeter (Hansatech Instruments Ltd, Norfolk, UK). Plants were dark adapted for 15 min before illuminating them for 1 sec while recording increase in fluorescence. The performance index PI(abs) was calculated from measured fluorescence. The activities of class III peroxidases (POD) and superoxide dismutases (SOD) were assessed by spectrophotometry (Cosio and Dunand, 2010; Regier et al., 2016). Lignin content was measured by thyoglycolic attack (Cosio and Dunand, 2010).

2.5. Cytosol extraction

Fresh plants were prepared as described in Regier et al. (2013b). Briefly, fresh plants were all collected at the same time, and weighed before being ground into 10 mL buffer (0.25M sucrose, 1 mM DTT, 50 mM Tris HCl pH 7.5). The resulting mixture was centrifuged at 500 g for 5 min at 4 °C. Supernatant, which contained cytosol, was collected and frozen until further used for analysis of metabolite concentrations. The dry mass of the whole shoots was calculated from the fresh biomass of each shoot weighed before the sap extraction based on previous observations that dry mass represents in average 18.3% of fresh mass for shoots.

2.6. Analysis of metabolites

For metabolomics, shoots of the plants exposed to the different experimental conditions for 24h were all collected at the same time, and immediately frozen in liquid nitrogen. Shoot were kept frozen at -30 °C until analysis. Three independent replicates, consisting of a pool of three shoots, or corresponding to the pooled cytosols of three plants (see section 2.5 for the method used for cytosol extraction), were run for each experimental condition. The plant samples were homogenized into 600 µL of ice-cold (-20 °C) methanol-chloroform solution (2:1, v:v) using a tungsten-bead beating apparatus (RetschTM MM301, Retsch GmbH, Haan, Germany) at 25 agitations per second for 1.5 min. Then, a volume of 400 µl of ice-cold ultra-pure water was added to each sample, and samples were subsequently vortexed and centrifuged at 4000 g for 10 min at 4 °C. A volume of 600 µL of the upper aqueous phase (which contained polar metabolites) was transferred to new microtubes, and further used for GC-MS and LC assays.

GC-MS Analyzes

Sixty (cytosol) or 120 (shoots) µL of the upper aqueous phase were transferred to new glass vials, and these aliquots were vacuum-dried (MiVac, Genevac Ltd., Ipswitch, England) at 32 °C for 45 min. Then, we used the derivatization process and GC-MS settings described in Khodayari et al., (2013).
Briefly, the dried aliquots were resuspended in 30 µl of 20 mg·L⁻¹ methoxyamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in pyridine, incubated under automatic orbital shaking at 40 °C for 90 min. Subsequently, 30 µl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Sigma) was added and the derivatization was conducted at 40 °C for 45 min under agitation. Our GC-MS system consisted of a Trace GC Ultra chromatograph and a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific, see Khodayari et al., 2013 for more details). Randomized sample sequences were established for sample injection, chromatograms were deconvoluted using XCalibur v2.0.7. Standard samples, consisting of 62 pure reference compounds at 1, 2, 5, 10, 20, 50, 100, 200, 500, 750, 1000, and 1500 µM were run, and metabolite levels were quantified using the quadratic calibration curves for each reference compound.

**LC analyzes**

Amino acid analysis was adapted from Renault et al. (2010) and (2016). Briefly, 100 µL of the metabolites in solution in the methanol-ultra-pure water extract was vacuum-dried (MiVac, Genevac Ltd., Ipswitch, England) at 32 °C for 30 min, and the pellet was re-suspended into 100 µL of ultra-pure water. Then, aliquots (10 µL) of the aqueous extracts were used for amino acids derivatization according to the AccQ•Tag ultra derivatization kit protocol (Waters Corporation, Milford, MA). Amino acids were analyzed using an Acquity UPLC® system (Waters Corporation, Milford, MA) by injecting 1 µL of the derivatization mix onto an Acquity UPLC® BEH C18 1.7 μm 2.1 x 100 mm column heated at 55 °C. External standards were run and used for the drawing of calibration curves, further allowing the quantification of the metabolites from the samples.

**2.7. Data Analysis**

Principal component analysis (PCA) was performed to identify patterns in response of variables and individuals, and compare the responses of the plants exposed to the three metal treatments, and the control. PCA were conducted with the packages FactoMineR (http://factominer.free.fr/) and ade4 (http://pbil.univ-lyon1.fr/ade4) in the R software (http://www.r-project.org). Normality and homoscedasticity were tested, and a student’s t-test was used in Excel (Microsoft, Redmond, WA, USA) to determine if two sets of data were significantly different. For the metabolic pathway analysis, the name of compounds that exhibited significant variations were analyzed in MetaboAnalyst (http://www.metaboanalyst.ca). The library of metabolic pathways was assembled from the KEGG database, and the significance of the pathway name was assessed with the Holm-Bonferroni method.
3. RESULTS

3.1. Bioaccumulation of metals in the plants and effects on physiological endpoints

The 24h-exposure of *E. nuttallii* to Cd, IHg and MeHg resulted in a significant bioaccumulation of metals in shoots (Table 1). Yet, this accumulation had no significant impact on measured physiological endpoints: lignin and chlorophyll contents, photosynthesis, POD and SOD activities, whose values were non-significantly different from control plants (Table 1).

3.2. Metabolites in shoots

We investigated the impact of short sublethal exposures to Cd, IHg and MeHg on the metabolic phenotypes of shoots of *E. nuttallii* (Table S1). In whole shoots, Cd treatment increased the concentrations of 11 amino acids, two sugars, one polyol (adonitol) and one organic acid (pipecolic acid), and reduced the amounts of two amino acids (aspartatic acid, methylcysteine), relative to control plants. Most strongly up-regulated amino acids by Cd included leucine, isoleucine and tryptophan showing a 16× increase, and tyrosine as well as valine showing 11× and 10× increase, respectively, after 24h of exposure to metals. Aspartatic acid and methylcysteine were reduced 0.05× and 0.3×, respectively by the 24h exposure to Cd. Similarly, MeHg exposure increased the amounts of ammonium and of 13 amino acids, and reduced the concentration of one polyol (arabitol 0.5×) (Table 2). Most strongly up-regulated amino acids included BABA (4×), tryptophan and methionine (2×). Exposure to IHg had no significant impact on concentrations of the measured metabolites in shoots, as compared with control plants.

Principal component analysis (PCA) was performed to compare metabolic profiles among treatments: 52% of the total variance was explained on axes 1 and 2 (Figure 1A and S1). In this PCA, Cd-exposed shoots showed the most distinct pattern, while centroids of IHg and MeHg-treated shoots were relatively similar to controls. Axis 1 was mainly constructed by the variations of adonitol, maltose, isoleucine, valine, tryptophan, tyrosine and leucine. Axis 2 was mainly supported by the variations of galactonolactone, glycerol, phosphoric acid, galactose, asparagine, glucose-6-phosphate and glyceric acid. In shoots, the metabolic pathway analysis performed thanks to KEGG databases revealed two biological metabolic pathways regulated by Cd: aminoacyl-tRNA biosynthesis, and valine, leucine and isoleucine biosynthesis. IHg had no significant impact at the level of pathways, while MeHg significantly impacted five metabolic pathways: aminoacyl-tRNA biosynthesis, glycine, serine and threonine metabolism, nitrogen metabolism, arginine and proline metabolism, cyanoamino acid metabolism.

3.3. Metabolites in the cytosol
Because amino acid and sugars transfer happens at the cellular level, we also investigated the impact of short sublethal exposures to Cd, IHg and MeHg on the metabolic phenotypes in the cytosol of *E. nuttallii*. Many amino acids are synthesized in the chloroplast and transported into the cytosol for protein and secondary metabolite synthesis, or transported and stored in the vacuole. Similarly, the transport of sugars and distribution of metabolites between compartments is coordinated in plants to drive growth and development. In the cytosol of plants, treatments significantly modified the concentration of several metabolites (Table 3): amounts of ammonium (1.7×), glycine (2.2×), hydroxyproline (1.6×) and phosphoric acid (2.2×) were increased by exposure to Cd. Concentrations of alpha-alanine (0.6×), aspartic acid (0.5×), galactose (0.4×), and mannose (0.3×) were reduced in Cd-exposed plants. Ammonium concentration rose by 1.6× in plants exposed to IHg, while this exposure reduced the amount of arginine (0.2×). In MeHg-exposed plants, changes of the concentration of individual amino acids, relative to controls, were significantly reduced for histidine (completely depleted) and arginine (0.2×). Regarding sugars, mannose was also reduced by IHg (0.6×) and MeHg (0.3×), and galactose by MeHg (0.3×). No significant variation was reported at the level of the metabolic pathways in the cytosol of the treated plants, as compared with control plants.

Principal component analysis of metabolites in cytosol explained 60% of the variance of samples on the first and second axes (Figure 1B and S2). Plants exposed to Cd showed the most distinct metabolic phenotypes relative to control plants on both axes 1 and 2. While MeHg-treated plants were separated from controls on axis 2, the centroid of IHg-treated plants was close to the one of their control counterparts. The first axis of the PCA was mainly supported by the variations of tryptophan, valine, proline, glutamic acid, phenylalanine, GABA and threonine. The second axis was constructed by the variations of glucose, galactose, mannose, alpha-alanine, inositol, aspartic acid and fructose.

4. DISCUSSION

4.1. Bioaccumulation and physiological endpoints

Both bioaccumulation and physiological endpoint analyses confirmed that metal concentrations used here resulted in their significant bioaccumulation, with sublethal effects for *E. nuttallii*, as earlier reported in other studies (Larras et al., 2013; Regier et al., 2013b; Beauvais-Fluck et al., 2018b). Chosen concentrations of metal in our work are representing a compromise between high concentrations resulting in unambiguous metal accumulation pattern in macrophytes, and concentrations with environmental relevance. Earlier investigations using the same concentrations of Cd (281 µg·L⁻¹) and IHg (70 ng·L⁻¹) for 7d revealed that root growth was reduced, while lignification
of stems was increased in *E. nuttallii* (Larras et al., 2013). Nevertheless, these treatments had no effects on chlorophyll content and shoot growth (Larras et al., 2013; Regier et al., 2013b). Conversely, exposure to MeHg (23 ng·L$^{-1}$) for 7d increased root growth, suggesting a hormesis effect that is seen as an overcompensation of a moderate stress (Larras et al., 2013). Similarly in another study, exposure for 2h to 10 ng·L$^{-1}$ or 10 µg·L$^{-1}$ MeHg had no significant effect on chlorophyll content, POD and SOD activities in *E. nuttallii* (Beauvais-Fluck et al., 2018b). In sum, consistent with the literature and based on the absence of effects of the physiological endpoints, our experimental conditions did not induce an acute stress in *E. nuttallii* shoots. Eventually, these data confirmed that *E. nuttallii* is highly tolerant to metals.

### 4.2. Effects of metals on the metabolic phenotypes of macrophytes

We were interested in obtaining a general picture of effects of toxic metals on the physiology of the plants by comparing their metabolic signatures. Our metabolic profiling revealed that all treatments impacted metabolite amounts in shoots, cytosol or both, as compared with control plants. The Cd-treated plants were characterized by the most distinct metabolic phenotypes according to PCA, in both shoots and cytosol. This finding highlights a higher impact of this metal on the amount of each metabolite, as also evidenced by a higher level of regulation (up to 16×) than the one measured in IHg and MeHg treatments. Even if this distinction may partially result from the higher Cd concentration used as compared with IHg and MeHg, we must re-emphasize that we did not find differences among the three treatments and the control for the other physiological endpoints. Moreover, metabolic pathway analysis suggested that MeHg impacted more metabolic pathways in shoots than did Cd in our experimental conditions. A previous proteomic study with identical experimental conditions suggested similar responses, and stress-induced level of IHg and Cd in *E. nuttallii*, and an absence of response for MeHg (Larras et al., 2013). Conversely, transcriptomic analyses suggested similar stress levels for Cd and IHg and a higher molecular impact of MeHg (Regier et al., 2013a; Beauvais-Fluck et al., 2018b). When examining responses of the two considered compartments (shoots and cytosol), we observed that Cd and MeHg both significantly impacted several pathways in shoots, while IHg had no significant impact on metabolites pathways. For cytosol, Cd had a higher impact than IHg, while MeHg had lower impact than IHg. Both cytosolic compartment analysis and proteomics target soluble molecules, suggesting that IHg might impact mainly soluble compartments, while MeHg impacts mainly the non-soluble compartments. However, these differences reflect different molecular toxicity pathways of the three metals, and highlight the interest of coupling analysis at different levels of organization. Indeed, the present study brings a different pattern as compared with those previously observed by transcriptomics. For instance,
MeHg was earlier reported as a stronger dysregulator than IHg, by affecting a much higher number of genes in *E. nuttallii* (Beauvais-Fluck et al., 2018b). In our study, metabolomics confirmed that MeHg has a stronger molecular effect than IHg in shoots, but not in cytosol. In addition, Cd, IHg, and MeHg regulated genes involved in biosynthesis of sulfur-containing amino acids such as methionine and cysteine (Regier et al., 2013a; Beauvais-Fluck et al., 2018a; Beauvais-Fluck et al., 2018b). Methionine was increased 2× by MeHg in shoots supporting its role in MeHg response, while cysteine was not altered after metal exposure in shoots or cytosol, suggesting that regulation at the gene and protein level was mostly happening to maintain the level of this metabolite.

### 4.3 Early signs of stress responses in *Elodea nuttallii* treated with Cd

Exposure of *E. nuttallii* resulted in a significant re-patterning of the metabolic phenotype, with for instance a significant effect on the concentrations of metabolites involved in aminoacyl-tRNA biosynthesis, and on the branched-chain amino acids. Aminoacyl-tRNA biosynthesis is involved in the synthesis of proteins in organisms, and are particularly central to growth (Banerjee et al., 2011; Raina and Ibba, 2014). Most often, the activity of this pathway declines when plants are exposed to stressful or restrictive conditions, as requirements for protein synthesis is reduced to favor the activation of stress-response genes (Holcik and Sonenberg, 2005). In parallel, we also found that Cd treatment impacted the concentration of branched-chain amino acids (*i.e.* leucine, isoleucine and valine) in shoots of *E. nuttallii*. Branched-chain amino acids share four enzymes for their biosynthesis, which are coordinately regulated. Plants that accumulate and tolerate high Cd amounts, such as *Solanum nigrum*, have been demonstrated to accumulate isoleucine and valine in roots (Xu et al., 2012). Branched amino acids derive from the shikimate pathway, and are required for protein synthesis and production of aromatic secondary metabolites, *e.g.* flavonoids, lignin cell wall components, and anthocyanins, which are important for cell wall extensibility and non-enzymatic anti-oxidative response both involved in metal tolerance in plants (Tzin and Galili, 2010; Zemanova et al., 2017). Branched-chain amino acids also serve as precursors for the production of secondary metabolites (*e.g.* alkaloids, glycosides) involved in responses to biotic and abiotic stress (Rizhsky et al., 2004). These secondary metabolites notably play a role during osmotic stress (Zemanova et al., 2017). Our metabolite profiling thus suggests that Cd exposure results in an osmotic stress to *E. nuttallii*, as depicted by the 10× to 16× increase of leucine, isoleucine and valine that typically accumulate in osmotically challenged plants. This hypothesis is further supported by the significant accumulation of proline, a well-known biomarker of water stress in plants. Indeed, proline can act as an osmolyte, a metal chelator, an antioxidative defense molecule and a signaling molecule (Hayat et al., 2012).
The probable osmotic stress induced by Cd-exposure likely increased the amount of oxidative stress experienced by the plants. Oxidative stress has been shown to alter the activity of the Krebs cycle (Obata and Fernie, 2012), with potential side effects on the pathway connected to the TCA, and thus on the levels of TCA-cycle-derived compounds. This includes aspartic acid, whose levels decreased in shoots (and to a lesser extent in the cytosol) of Cd-treated plants. Besides, we also measured decreased amounts of mannose and galactose in shoots of *E. nuttalli*. These two sugars are involved in the Smirnoff-Wheeler pathway, and their reduction suggests that they were used for the biosynthesis of ascorbic acid. Ascorbic acid is widely recognized as an important ROS scavenger in plants, and earlier studies reported that accumulation or enhanced production of ascorbic acid boosted the ability of plants to cope with salt or drought stress, by limiting the peroxidation of membrane lipids and reducing losses of the chlorophyll content (Venkatesh and Park, 2014). Increased ROS production in Cd-treated plant is very likely, as this is a reported toxic effect of metal exposure at higher concentrations (Kieffer et al., 2009; Xu et al., 2012; Larras et al., 2013).

Finally, the accumulation of leucine, isoleucine and valine may serve to promote stress-induced protein synthesis, and may act as signaling molecules to regulate gene expression (Joshi et al., 2010). The breakdown products of these three amino acids, *e.g.* acetyl-CoA, propionyl-CoA, and acetoacetate, are potential energy sources for plants (Joshi et al., 2010). The global increase of numerous amino acids likely depicts increased degradation and synthesis of proteins (Vital et al., 2017). Similar observation was reported in crop cultivars, and shown to be linked to impact of osmotic stress on the proteasome (Vital et al., 2017).

4.4. MeHg affects the nitrogen metabolism and triggers chemical defenses against abiotic stress

In macrophytes exposed 24h to MeHg, the amount of several amino acids, including aspartic acid, glutamine, glycine, phenylalanine, proline, threonine and tryptophan were altered in shoots. This finding is congruent with previous transcriptomic studies conducted on *E. nuttallii* exposed to MeHg, which showed that genes involved in synthesis of proteins represented a high proportion of regulated genes (Beauvais-Fluck et al., 2018a). Altogether, data suggest a rapid adjustment of the plants to MeHg exposure. Several of these amino acids are expected to have a role in chemical defenses against abiotic stresses, notably glycine, threonine and proline (as discussed above).

Our metabolomic data, and more particularly the significant rise of glutamine and ammonium amounts, further support that MeHg affects nitrogen (N) metabolism and homeostasis in the plant. The increase of the amount of aspartatic acid and glutamine in MeHg-treated plants, and the concomitant reduction of ammonium amount in cytosol, suggest that N transport is altered. In
plants, the main pathway of ammonia assimilation is the GS/GOGAT cycle, where glutamine synthetase / glutamate synthase cooperate (Masclaux-Daubresse et al., 2006). Plants may also utilize the reaction catalyzed by glutamate dehydrogenase for ammonia assimilation, especially under environmental stress conditions (Masclaux-Daubresse et al., 2006). Consequently, in plants, aspartic acid, asparagine, glutamic acid and glutamine are the main amino acids transporter of N (Miesak and Coruzzi, 2002). Here, the increase in ammonia and glutamine (high N/C ratio) suggest that nitrate assimilation is affected, and both ammonia and amino acids are released by protein degradation and hydrolysis. Similarly, previous transcriptomic study in E. nuttallii exposed to IHg, MeHg or Cd highlighted a down-regulation of genes coding for nitrate and ammonium transport (e.g. high affinity nitrate transporters) suggesting a global impact of tested metals on nutrition (Regier et al., 2013a; Beauvais-Fluck et al., 2018a).

Phenylalanine, threonine, and tryptophan are aromatic amino acids coupled with the phenylpropanoid and the shikimate pathways. Phenylpropanoids are a diverse group of compounds derived from the carbon skeleton of phenylalanine. These compounds are involved in plant defense and generate cell wall components (e.g. lignin) and antioxidant metabolites (e.g. anthocyanin) (Fraser and Chapple, 2011). In previous study, a significant increase of anthocyanin, acting as non-enzymatic antioxidants (Beauvais-Fluck et al., 2018a; Beauvais-Fluck et al., 2018b), was measured in E. nuttallii exposed 2h to 10 ng·L⁻¹ MeHg and higher concentrations, but not after lHg treatments (Beauvais-Fluck et al., 2018b). The increase amount of phenylalanine, threonine, and tryptophan suggests enhanced antioxidant activity in MeHg-treated plants.

4.5. Toxic metals had little effects on the concentration of sugars

A previous study hypothesized that the accumulation of fructose, galactose, sucrose and glucose in poplar (Populus tremula L.) exposed to Cd might act as osmoprotectants playing critical roles in osmotic adjustments, and protection of cell membranes against toxic trace metals (Kieffer et al., 2009). Here, exposures decreased mannose and galactose amounts in cytosol. Both sugars are involved in the ascorbic acid biosynthetic pathway, a major antioxidant in higher plants, and their decrease is thus in line with expected cellular toxicity of metals (Ma et al., 2014). Other studies suggested cellular adjustments to control resources distribution within cells: reduced sugar utilization in the presence of Cd and consequently photoassimilates partial storage as sugars (Moya et al., 1995; Bailey et al., 2003). In the aquatic plant Wolffia arrhizal, sugar and protein contents were negatively correlated with toxic metal concentrations (Piotrowska et al., 2010; Zhang et al., 2017). In the same line, sugars accumulated in correlation with a decline of net photosynthetic rates in rice exposed to Cd (Moya et al., 1995). Similarly, amino acid metabolism often is expected to be
closely related to abiotic stress tolerance (Zhang et al., 2017). However, in our study few sugars varied between treated and control plants, both in cytosol and shoots. Nevertheless, we observed an increase of sugars in shoots, while their content concomitantly decreased in cytosol. The transport of sugars and distribution between storage, respiration and biosynthesis in sink tissues are coordinated activities in plants (Rosa et al., 2009a; Lemoine et al., 2013). Stress-related increase in sugar delivery to sinks is important for growth, cell turgor and water potential maintenance (Lemoine et al., 2013). Besides, soluble sugars have several roles in cells, including as metabolic resources, structural constituents of cells, but also in stress signaling (Rosa et al., 2009b). Transporters are required for efficient movement of sugars that are polar solutes across membranes (Patrick et al., 2013). It is likely that multiple sugar transporters operate for fine-tuning of sugar fluxes for homeostasis and interaction with other proteins for sugar sensing and signaling, including efficient unloading into cell wall spaces, uptake of sucrose or transport via the apoplasm. However, metal exposure concentrations used here might be too low to impact significantly sugar metabolic pathways. Metabolic data thus support that previously observed regulation of genes and proteins involved in sugar metabolism was mostly happening to maintain the level of those metabolites (Regier et al., 2013a; Beauvais-Fluck et al., 2018a; Beauvais-Fluck et al., 2018b).

5. CONCLUSION
Metabolomic analysis showed that exposure of *E. nuttallii* to Cd, IHg and MeHg resulted in different metabolomes in each compartment (shoot and cytosol) with some level of coordinated regulation for metabolites between shoots and cytosol. Metabolomic confirmed previous hypothesis of distinct toxicity pathway for IHg and MeHg based on transcriptomics, proteomics and physiology (Larras et al., 2013; Regier et al., 2013a; Beauvais-Fluck et al., 2018a). In contrast to previous proteomics and transcriptomics data, Cd showed a higher impact on individual metabolites, while MeHg impacted more metabolic pathways in shoots, but IHg showed a higher impact in cytosol than MeHg. New omics methods are fundamentally transforming biology approaches, but the fact that many genes and proteins identified by transcriptomics and proteomics have an unknown function results in considerable uncertainty in the analysis of responses. Metabolomics give the opportunity to confirm and reveal effects of toxicants in a complementary way to other omics methods, as it identified early responses that were effective in revealing metal uptake and impact as well as were congruent with expected adverse outcome pathways.

ACKNOWLEDGMENT
Experiments were performed at Geneva University during CC previous position. Authors thank
Rebecca Beauvais-Fluck, Floriane Larras, Beatriz Lobo, Nicole Regier and Debora Tanaami for their help in the management of cultures, sampling of water and the preparation of cytosol extracts.

FUNDINGS

The Swiss National Science Foundation (contracts n°205321_138254 and 200020_157173).

REFERENCES


Bonanno, G., Lo Giudice, R., 2010. Heavy metal bioaccumulation by the organs of Phragmites australis (common reed) and their potential use as contamination indicators. Ecological Indicators 10, 639-645.


Lemoine, R., La Camera, S., Atanasssova, R., Dedaldechamp, F., Allario, T., Pourtau, N., Bonnemain, J.-L., Laloi, M., Coutos-Thevenot, P., Maurousset, L., Faucher, M., Girousse, C., Lemonnier, P., Parrilla,


exposed to cadmium and lead. Archives of Environmental Contamination and Toxicology 58, 594-604.


Table 1. Lignin content, chlorophyll content, performance index of PSII \( [\text{PI}(\text{abs})] \), class III peroxidase \( [\text{POD}] \) and superoxide dismutase \( [\text{SOD}] \) activities and bioaccumulation in shoots of *Elodea nuttallii* exposed to 280 \( \mu \text{g·L}^{-1} \) Cd, 70 ng·L\(^{-1}\) IHg, or 30 ng·L\(^{-1}\) MeHg or not (control). For each treatment, three replicates were analyzed. Data are presented as mean ± standard deviation (SD; \( n=3 \)). Bold font = \( p \)-value <0.05 relative to control plants.

<table>
<thead>
<tr>
<th>Shoots</th>
<th>Bioaccumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lignin content</td>
<td>Chlorophyll</td>
</tr>
<tr>
<td>(µg·mg(^{-1}) fresh mass)</td>
<td>(nkat·mg(^{-1}) protein)</td>
</tr>
<tr>
<td>Control</td>
<td>7.2 ± 1.3</td>
</tr>
<tr>
<td>Cd</td>
<td>6.4 ± 1.4</td>
</tr>
<tr>
<td>IHg</td>
<td>5.6 ± 1.3</td>
</tr>
<tr>
<td>MeHg</td>
<td>5.6 ± 2.2</td>
</tr>
</tbody>
</table>

NA (not analyzed).
Table 2. Concentration (nmoles·mg⁻¹ dry mass) of amino acids, organic acids, polyols and sugars (n=3; mean ± SD) whose levels significantly varied in shoots of *E. nuttallii* exposed for 24h to Cd, IHg and MeHg (bold font = p-value <0.05), relative to control plants.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cd</th>
<th>IHg</th>
<th>MeHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium</td>
<td>4.21 ± 1.03</td>
<td>6.12 ± 2.35</td>
<td>3.90 ± 0.89</td>
<td><strong>7.72 ± 2.06</strong></td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>15.22 ± 2.79</td>
<td><strong>0.72 ± 0.84</strong></td>
<td>13.41 ± 2.44</td>
<td><strong>21.77 ± 1.77</strong></td>
</tr>
<tr>
<td>BABA</td>
<td>0.02 ± 0.01</td>
<td><strong>0.09 ± 0.06</strong></td>
<td>0.07 ± 0.05</td>
<td><strong>0.08 ± 0.06</strong></td>
</tr>
<tr>
<td>Glutamine</td>
<td>9.03 ± 2.06</td>
<td>6.73 ± 1.61</td>
<td>8.87 ± 2.13</td>
<td><strong>14.61 ± 4.43</strong></td>
</tr>
<tr>
<td>Glycine</td>
<td>1.20 ± 0.20</td>
<td>1.00 ± 1.43</td>
<td>0.97 ± 0.23</td>
<td><strong>1.96 ± 0.30</strong></td>
</tr>
<tr>
<td>Histidine</td>
<td>0.38 ± 0.12</td>
<td><strong>1.36 ± 0.45</strong></td>
<td>0.30 ± 0.07</td>
<td>0.47 ± 0.19</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0.00 ± 0.00</td>
<td>0.04 ± 0.04</td>
<td>0.01 ± 0.01</td>
<td><strong>0.02 ± 0.01</strong></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.24 ± 0.17</td>
<td><strong>3.73 ± 1.14</strong></td>
<td>0.15 ± 0.12</td>
<td>0.40 ± 0.24</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.17 ± 0.08</td>
<td><strong>2.74 ± 0.70</strong></td>
<td>0.12 ± 0.05</td>
<td><strong>0.28 ± 0.08</strong></td>
</tr>
<tr>
<td>Lysine</td>
<td>0.25 ± 0.21</td>
<td><strong>1.22 ± 0.42</strong></td>
<td>0.18 ± 0.18</td>
<td>0.41 ± 0.34</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.03 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td><strong>0.06 ± 0.01</strong></td>
</tr>
<tr>
<td>Methylcysteine</td>
<td>0.10 ± 0.05</td>
<td><strong>0.03 ± 0.03</strong></td>
<td>0.07 ± 0.03</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.38 ± 0.08</td>
<td><strong>2.15 ± 0.78</strong></td>
<td>0.34 ± 0.03</td>
<td><strong>0.66 ± 0.15</strong></td>
</tr>
<tr>
<td>Proline</td>
<td>0.39 ± 0.15</td>
<td><strong>1.93 ± 0.46</strong></td>
<td>0.30 ± 0.13</td>
<td><strong>0.64 ± 0.23</strong></td>
</tr>
<tr>
<td>Serine</td>
<td>4.49 ± 1.05</td>
<td>4.64 ± 0.56</td>
<td>4.29 ± 1.03</td>
<td><strong>7.21 ± 1.02</strong></td>
</tr>
<tr>
<td>Threonine</td>
<td>1.91 ± 0.37</td>
<td><strong>5.68 ± 1.72</strong></td>
<td>1.73 ± 0.35</td>
<td><strong>3.08 ± 0.47</strong></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.10 ± 0.02</td>
<td><strong>1.60 ± 0.33</strong></td>
<td>0.12 ± 0.05</td>
<td><strong>0.21 ± 0.03</strong></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.14 ± 0.04</td>
<td><strong>1.54 ± 0.38</strong></td>
<td>0.12 ± 0.03</td>
<td><strong>0.24 ± 0.03</strong></td>
</tr>
<tr>
<td>Valine</td>
<td>0.52 ± 0.28</td>
<td><strong>5.24 ± 1.44</strong></td>
<td>0.37 ± 0.24</td>
<td>0.82 ± 0.41</td>
</tr>
<tr>
<td>Adonitol</td>
<td>0.45 ± 0.09</td>
<td><strong>1.07 ± 0.31</strong></td>
<td>0.46 ± 0.07</td>
<td>0.44 ± 0.08</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.65 ± 0.20</td>
<td><strong>0.95 ± 0.19</strong></td>
<td>0.59 ± 0.09</td>
<td>0.47 ± 0.07</td>
</tr>
<tr>
<td>Arabinol</td>
<td>0.28 ± 0.04</td>
<td>0.23 ± 0.04</td>
<td>0.18 ± 0.04</td>
<td><strong>0.15 ± 0.03</strong></td>
</tr>
<tr>
<td>P襟jepolic Acid</td>
<td>0.65 ± 0.20</td>
<td><strong>0.95 ± 0.19</strong></td>
<td>0.59 ± 0.09</td>
<td>0.47 ± 0.08</td>
</tr>
<tr>
<td>Ribose</td>
<td>2.81 ± 1.02</td>
<td><strong>5.43 ± 1.39</strong></td>
<td>3.02 ± 0.74</td>
<td>3.46 ± 1.19</td>
</tr>
</tbody>
</table>
Table 3. Concentration (nmoles·mg⁻¹ dry mass) of amino acids, organic acids, polyols and sugars (n=3; mean ± SD) whose amounts significantly varied (relative to control) in the cytosol of *E. nuttallii* exposed for 24h to Cd, IHg and MeHg (bold font = p-value <0.05).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cd</th>
<th>IHg</th>
<th>MeHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-alanine</td>
<td>0.48 ± 0.01</td>
<td><strong>0.31 ± 0.05</strong></td>
<td>0.50 ± 0.17</td>
<td>0.33 ± 0.18</td>
</tr>
<tr>
<td>Ammonium</td>
<td>0.52 ± 0.13</td>
<td><strong>0.89 ± 0.11</strong></td>
<td><strong>0.81 ± 0.07</strong></td>
<td>0.62 ± 0.32</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.16 ± 0.02</td>
<td>0.05 ± 0.06</td>
<td><strong>0.02 ± 0.01</strong></td>
<td><strong>0.04 ± 0.04</strong></td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>1.22 ± 0.12</td>
<td><strong>0.60 ± 0.11</strong></td>
<td>1.23 ± 0.41</td>
<td>0.77 ± 0.44</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.18 ± 0.03</td>
<td><strong>0.40 ± 0.03</strong></td>
<td>0.12 ± 0.03</td>
<td>0.19 ± 0.15</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.14 ± 0.01</td>
<td>0.58 ± 0.99</td>
<td>0.07 ± 0.01</td>
<td><strong>0.0 ± 0.0</strong></td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>3.68 ± 0.25</td>
<td><strong>5.89 ± 0.84</strong></td>
<td>4.42 ± 0.83</td>
<td>3.84 ± 2.01</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.99 ± 0.01</td>
<td><strong>0.39 ± 0.03</strong></td>
<td>0.63 ± 0.21</td>
<td><strong>0.35 ± 0.17</strong></td>
</tr>
<tr>
<td>Mannose</td>
<td>0.08 ± 0.01</td>
<td><strong>0.02 ± 0.01</strong></td>
<td><strong>0.04 ± 0.02</strong></td>
<td><strong>0.03 ± 0.01</strong></td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>6.89 ± 1.26</td>
<td><strong>15.20 ± 3.09</strong></td>
<td>7.31 ± 0.82</td>
<td>7.26 ± 3.35</td>
</tr>
</tbody>
</table>
Figure 1. Principal component analysis of amino acids, organic acids, sugars and polyols measured in shoots (A) and cytosol (B) of *E. nuttallii* exposed 24h to 281 µg·L⁻¹ Cd, 70 ng·L⁻¹ lHg and 30 ng·L⁻¹ MeHg, and their control (ctl) (n=6 and 3 ± SD, respectively). PCA were calculated and plotted with the package ade4 (http://pbil.univ-lyon1.fr/ade4) in the R software (http://www.r-project.org).
Cd, IHg and MeHg effects were compared in shoots and cytosol by metabolomics

In shoots, Cd showed higher regulation, but MeHg regulated more pathways

In cytosol, Cd had the higher impact, IHg had low effect and MeHg had very low effect

MeHg impacts N homeostasis, and Cd results in an osmotic stress-like pattern

Metabolomics support different molecular toxicity pathways for the three metals
Authors declare no conflict of interest