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Research Paper

Effects of two-hour exposure to environmental and high concentrations of methylmercury on the transcriptome of the macrophyte *Elodea nuttallii*

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ABSTRACT

The effects of two methylmercury (CH_3Hg^+, MeHg) concentrations, representative of environmental level and extreme contamination, were investigated on the macrophyte *Elodea nuttallii* during a 2h-exposure combining transcriptomic (RNA-Seq), physiological endpoints (pigment contents, activity of anti-oxidative stress enzymes) and bioaccumulation. Exposure to MeHg induced the up- and down-regulation of numerous genes (4389 and 16853 for 10 ng L^{-1} and 10 µg L^{-1} MeHg exposure, respectively) involved in sugar, amino acid and secondary metabolism (e.g. cinnamic acid, flavonoids) at both concentrations. Genes coding for photosynthesis, membrane integrity, metal homeostasis, water transport and anti-oxidative enzymes were additionally up- and down-regulated at the higher concentration. At the physiological level, exposure to both MeHg concentrations resulted in a strong increase of anthocyanin content in shoots. Chlorophyll content and antioxidant enzyme activities were unchanged. The data suggest that the macrophyte was able to efficiently cope with the stress resulting from MeHg exposure, possibly by using anthocyanin as anti-oxidant and S-rich amino acids (such as cysteine and methionine) as chelators. Transcriptomics analysis enabled gaining novel insights on molecular effects of MeHg in primary producers, which are one of the main entry pathway of hazardous MeHg in aquatic food webs.

1. Introduction

Mercury compounds are of concern for current global environmental quality and risk management of aquatic ecosystems. Both inorganic (IHg, Hg^{2+}) and organic mercury (i.e. methyl mercury, MeHg, CH_3Hg^{+},) bioaccumulate and can deleteriously affect the biota. Moreover, MeHg strongly biomagnifies in the trophic chain and subsequently has a potential to affect top chain consumers (higher predators, humans) (Clarkson and Magos, 2006; Clayden et al., 2016; Tom et al., 2010). Nevertheless, to date there has been limited success in directly linking the water chemistry with the uptake of Hg compounds and MeHg biomagnification in food webs. Macrophytes are aquatic primary producers which easily accumulate both IHg and MeHg, and play a key role in the fate of Hg in shallow aquatic environments, notably as an entry point in food webs (Beauvais-Fluck et al., 2016a, 2017a; Bravo et al., 2014; Gentes et al., 2013; Larras et al., 2013; Regier et al., 2012). Understanding the uptake mechanisms of Hg and its toxicity in macrophytes is therefore instrumental to understand the fate of Hg and to better evaluate its risk in the ecosystems.

Elodea nuttallii is an invasive rooted-submerged macrophyte widely spread in shallow fresh waters, recently identified because of its high accumulation of Hg from both the sediment and the water column and further Hg remobilization to herbivores (Beauvais-Fluck et al., 2016a, 2017a; Bravo et al., 2014; Larras et al., 2013; Regier et al., 2012). A proteomic and physiological study of *E. nuttallii* exposed for 24 h to 70 ng L^{-1} IHg pointed to several tolerance mechanisms: (i) an acclimation of the cell structure (e.g. lignin synthesis, cytoskeleton organization) and (ii) modification of the energy metabolism (e.g. sugar catabolism, photosynthesis) (Larras et al., 2013). Another recent analysis of effects of IHg through whole transcriptome analysis in *E. nuttallii* exposed 24 h to 200 ng L^{-1} IHg similarly revealed the up- and down-regulation of genes involved in the energy metabolism (e.g. sugar catabolism, photosynthesis), in the stress responses (e.g. genes coding for chaperones, heat-shock proteins), and in the uptake of nutrient (e.g. genes coding for essential metal transporters) (Regier et al., 2013a). These results revealed that oxidative stress and alteration of protein conformation are involved in the mechanism of IHg toxicity. In the same line, exposure for 24 h to 10 µg L^{-1} IHg of the submerged macrophyte *Potamogeton crispus* induced lipid peroxidation (Ali et al., 2000), while 24 h-exposure to 2 mg L^{-1} IHg inhibited nutrient

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assimilation of the submerged macrophyte *Vallisneria spiralis* (Gupta and Chandra, 1998) and modified cell wall metabolism of alfalfa *Medicago truncatula* (Zhou et al., 2013). Other reported effects included reduction of root growth of *Medicago sativa* exposed 72 h to 1 mg L^{-1} IHg (Zhou et al., 2007) and senescence of 3 submerged angiosperms exposed 3d to 2 g L^{-1} IHg (Jana and Choudhuri, 1982). By contrast, the toxicity of MeHg has rarely been assessed in an aquatic plant in comparison with other organisms. One earlier study reported that 24 h-exposure to 23 ng L^{-1} MeHg on *E. nuttallii* did not result in significant response at the proteomic nor at the physiological level (Larras et al., 2013). Nonetheless, identification of intracellular targets and molecular effects of MeHg on macrophytes are central to better predict the fate of MeHg in food webs.

The present study therefore aims at increasing the mechanistic understanding on the intracellular targets and effects of MeHg on a representative macrophyte, *E. nuttallii*. The transcriptomic response (RNA-Seq), bioaccumulation and physiological effects in *E. nuttallii* exposed for 2 h to 10 ng L^{-1} and 10 µg L^{-1} MeHg were measured simultaneously. This short-time exposure was chosen to target early-response genes to MeHg, before more general stress response appears. The two MeHg concentrations were chosen to represent environmental and extreme pollution. The lower concentration (10 ng L^{-1} MeHg) is representative of environmental concentrations below the European environmental quality standard for Hg (50 ng L^{-1} Hg) (Bravo et al., 2014; Crane and Babut, 2007), while the higher concentration (10 µg L^{-1} MeHg) may occur in an acute exposure scenario. Indeed, a 4-day-long exposure to 300 ng L^{-1} MeHg reduced root growth in *E. nuttallii* grown and exposed under similar condition as here (Larras et al., 2013). Therefore, we expected the higher concentration of MeHg to strongly alter the plant metabolism.

2. Experimental section

2.1. Lab-ware and reagents

All material was soaked in 10% v/v HNO_3 (*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. Methylmercury chloride standard stock solution (1 g L^{-1}) was obtained from Alfa Aesar (Ward Hill, MA, USA).

2.2. Plant culture and exposure conditions

Shoots of *Elodea nuttallii* were collected in Lake Geneva, and a culture established and maintained in microcosms as previously described (Regier et al., 2013b). Cultures and experiments were conducted in the laboratory under the same controlled conditions (16/8 h with 5.84 W m^{-2} photosynthetically active radiation (PAR), 1000 lux; 20 ± 1 °C). Exposures were conducted in triplicates and consisted of exposing three 10 cm-long shoots without roots to 10 ng L^{-1} or 10 µg L^{-1} MeHg in 500 mL artificial medium (91.0 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 43.2 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 23.5 mg L^{-1} $NaHCO_3$, 13.6 mg L^{-1} KH_2PO_4 and 0.4 mg L^{-1} NH_4NO_3, pH 6.9 ± 0.1) during 2 h. Initial concentrations of MeHg and total Hg (THg = IHg + MeHg) in the exposure medium were measured by MERX® (see below). In these experimental conditions, no demethylation occurred in the medium (Larras et al., 2013).

2.3. Mercury bioaccumulation in E. nuttallii

At the end of the exposure, half of the shoots were washed 10 min with 292 mg L^{-1} ethylenediaminetetraacetic acid (EDTA, ACS reagent, Sigma-Aldrich, Buchs, Switzerland) and 121 mg L^{-1} cysteine (Cell Culture Tested, Sigma-Aldrich, Buchs, Switzerland). This washing procedure has been shown to be the most efficient to wash out Hg loosely bound to the cell wall and hence allows estimating intracellular Hg content, although it slightly overestimated intracellular MeHg due to the high affinity of MeHg to proteins in cell walls (Larras et al., 2013). The unwashed shoots were used to measure Hg accumulated in the whole shoot (= intracellular + adsorbed Hg). Adsorbed Hg was estimated by subtracting intracellular Hg from Hg in unwashed shoots (= whole shoots − rinsed shoots). All samples were then rinsed in ultrapure water, carefully dried and frozen at −80 °C until further treatment.

To measure THg contents, shoots were freeze-dried (Beta 1–8 K freeze dryer, Christ, Germany) and analyzed with an advanced mercury analyzer (AMA 254, Altec, Dvůr Králové nad Labem, Czech Republic) (USEPA, 1998). The certified reference material (CRM) MESS-3 (National Research Council of Canada) was used to assess the accuracy of analysis.

To measure MeHg contents, shoots were ground in liquid nitrogen, freeze-dried, mineralized 12 h at 60 °C in 30% v/v HNO_3 (Suprapur®, Merck Millipore, Darmstadt, Germany) and analyzed after appropriate dilution by cold vapor fluorescence spectrometry with a MERX® Automated Methylmercury Analytical System (Brooks Rand Instruments, Seattle, WA, USA) following the manufacturer's instructions and standardized protocol (USEPA, 2001). The Tort-2 (National Research Council of Canada) was used as CRM.

The concentration in the unexposed shoots was 0.022 ± 0.009 µg g^{-1} MeHg dry weight (DW; MeHg = 34% of THg). Background concentration in the medium was 0.0239 ± 0.0023 ng L^{-1} MeHg (MeHg = 0.7% of THg). Measured MeHg concentrations (MeHg = 100% of THg) in the exposure medium were 7.72 ± 1.10 ng L^{-1} MeHg for 10 ng L^{-1} MeHg treatment and 10.01 ± 0.50 µg L^{-1} MeHg for 10 µg L^{-1} MeHg treatment. Under the studied conditions, MeHg chemical speciation included 64.9% CH_3HgOH^0, 34.7% CH_3HgCl^0, 0.3% CH_3Hg^+, and 0.1% others, calculated by using WHAM/ModelVII with updated constant for MeHg (Tipping et al., 2011).

2.4. Transcriptomic response to MeHg

The response at the transcriptome level in *E. nuttallii* was determined in triplicate in unexposed control and MeHg exposed shoots. At the end of the exposure, shoots were quickly rinsed in ultrapure water, snap-frozen and ground in liquid nitrogen, and stored at −80 °C. Total RNA was extracted using TRI Reagent® (Sigma-Aldrich, Buchs, Switzerland) following provider's instructions. RNA concentration was determined by Qubit (Life technologies, Zug, Switzerland) and quality was assessed by agarose gel electrophoresis and LabChip GX/GX II (PerkinElmer, Inc., Wellesley, Mass., USA).

2.4.1. Libraries sequencing, de novo transcriptome assembly and mapping

Libraries were prepared and sequenced using HiSeq 2000 technology (Illumina, San Diego, CA, USA) following the manufacturer instructions. Single-reads with a length of 100 bp were obtained, and *de novo* assembling realized using the module Oases (version 0.2.08; http://www.ebi.ac.uk/~zerbino/oases/) (Schulz et al., 2012) in Velvet (version 1.2.07; http://www.ebi.ac.uk/~zerbino/velvet/) (Zerbino and Birney, 2008). The best assembly was selected using the Burrows-Wheeler Alignment tool BWA (V0.5.9-r16). Mapping statistics ranged from 69.7 to 73.2% for Control and MeHg samples. The *de novo* transcriptome contained 181′663 contigs, with an average length of 880 bp. The RNA-Seq data are deposited in the NCBI public database (PRJNA245505).

2.4.2. Differential gene expression analysis

Reads were counted using MAQ (Mapping and Assembly with Qualities; version 0.7.1) (Li and Durbin, 2010). We selected the contigs showing a minimum coverage of 20 raw counts in all samples, resulting