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Towards early-warning gene signature of *Chlamydomonas reinhardtii* exposed to Hg-containing complex media

Rébecca Beauvais-Flück[1], Vera I. Slaveykova[1], Skyllberg Ulf[2], Claudia Cosio[1]*†

[1]Department F.-A. Forel for environmental and aquatic sciences, Earth and Environmental Sciences, Faculty of Sciences, University of Geneva, 66, boulevard Carl-Vogt, CH-1211 Geneva 4, Switzerland.

[2]Department of Forest Ecology and Management, Swedish University of Agricultural Sciences, 901 83 Umeå, Sweden

†present address: Unité Stress Environnementaux et BIOSurveillance des Milieux Aquatiques UMR-I 02 (SEBIO), Université de Reims Champagne Ardenne, F-51687 Reims, France.

*Corresponding authors: claudia.cosio@unige.ch, claudia.cosio@univ-reims.fr

Abstract

The potential of using gene expression signature as a biomarker of toxicants exposure was explored in the microalga *Chlamydomonas reinhardtii* exposed 2 hours to mercury (Hg) as inorganic mercury (IHg) and methyl mercury (MeHg) in presence of copper (Cu) and Suwannee River Humic Acid (SRHA). Total cellular Hg (THg = IHg + MeHg) decreased in presence of SRHA for 0.7 nM IHg and 0.4 nM MeHg, but increased for 70 nM IHg exposure. In mixtures of IHg + MeHg and (IHg or MeHg) + Cu, SRHA decreased THg uptake, except for 0.7 nM IHg + 0.4 nM MeHg which was unchanged. In the absence of SRHA, 0.5 μM Cu strongly decreased intracellular THg concentration for 70 nM IHg, while it had no effect for 0.7 nM IHg and 0.4 nM MeHg. The expression of single transcripts was not correlated with measured Hg uptake, but a subset of 60 transcripts showed signatures specific to the exposed metal(s) and was congruent with exposure concentration. Notably, the range of fold change values of this subset correlated with THg bioaccumulation with a two-slope pattern in line with $[\mathrm{THg}]_{\mathrm{intra}}/[\mathrm{THg}]_{\mathrm{med}}$ ratios. Gene expression signature seems a promising approach to complement chemical analyses to assess bioavailability of toxicants in presence of other metals and organic matter.

Keywords: copper, dissolved organic matter, microalgae, uptake, transcriptomics.

1. Introduction

For environmental risk assessment, a rapid diagnostic is instrumental to limit pollution impacts. Thus, the development of early warning tools is highly desirable. In this context, managers of ecosystems use ecotoxicology - the study of biota responses to toxicants- to evaluate the level of toxicity of pollution to identify the most efficient actions. Historically ecotoxicology focused on cellular effects, such as growth or photosynthesis efficiency. Recent advances in system biology, notably new genomic sequencing techniques are fundamentally transforming ecotoxicology approach by offering powerful tools to directly detect the earliest stages of the toxicological response including non-models species with unsequenced genomes (Beauvais-Fluck et al., 2016, 2017; Beauvais-Fluck et al., 2018b; Brinke and Buchinger, 2017; Regier et al., 2016). Transcriptomic offers a great potential because it was shown to be efficient for analysis of short-term exposure, more sensitive than classical bioassays (e.g. bioaccumulation or physiological effects) and to correlate with gradients of contaminants in natural waters, as well as to be able to identify toxicant-specific signatures (Dranguet et al., 2017a; Garcia-Reyero et al., 2009; Gomez-Sagasti et al., 2016; Regier et al., 2013a; Yang et al., 2007). Indeed, several toxicological studies were able to differentiate toxicants on the basis of the gene expression profiles in exposed organisms to multiple environmental stressors, offering a more thorough analysis than currently available bioassays (Aardema and MacGregor, 2002; Beauvais-Fluck et al., 2018a; Beauvais-Fluck et al., 2018b; Poynton et al., 2011; Regier et al., 2013a; Waring et al., 2001). Moreover, transcriptomic has the potential of identifying the impact of several stressors in a single analysis and seems hence particularly interesting for *in-situ* analysis characterized by a cocktail of different metals and the presence of organic matter (Almeida et al., 2005; Beauvais-Fluck et al., 2018a; Beauvais-Fluck et al., 2018b; Dondero et al., 2011; Hutchins et al., 2010; Milan et al., 2015; Regier et al., 2016; Villeneuve et al., 2012). However, there is now a need to better evaluate the potential of this tool, in particular its predictive aspects of bioavailability and toxicity to the ecosystem (Fedorenkova et al., 2010). Notably, the connection between gene response and environmental exposure needs to be investigated in more detail.

Mercury (Hg) toxicity and biomagnification in trophic web is a worldwide hazard in aquatic ecosystems (Lavoie et al., 2013). Nonetheless, an efficient early-warning tool to reliably assess Hg bioavailability and its potential impact in natural environments is still missing. Because Hg enters the food web through phytoplankton (Bravo et al., 2014), microalgae are key organisms to assess Hg exposure (Le Faucheur et al., 2014). In the environment, microalgae are exposed to Hg in the presence of other metals and metal binding organic ligands. In aquatic environments Hg occurs as inorganic Hg (IHg) and methyl Hg (MeHg) and the concentration of total Hg (THg = IHg + MeHg) generally spans between 1 pM to 30 nM, with MeHg representing 1 to 30% of THg (Bravo et al., 2014; Cossa et al., 2009). Currently, the European environmental quality standard for freshwater protection is 0.35 nM THg (Crane and Babut, 2007). What is more, the dissolved organic matter (DOM) present in freshwaters is considered as an important environmental factor that protects the aquatic primary producers from metal stress. Indeed DOM functional groups play a key role for the bioavailability of Hg and MeHg to microalgae by dictating the chemical speciation of Hg (Skyllberg, 2011). Further, the complex interplay between DOM, Hg and other soft metals, also affects the impact of Hg on cells by interacting on similar cellular targets and/or indirectly affecting Hg uptake (Beauvais-Fluck et al., 2018b; Ravichandran, 2004). Because of this complexity, the impact of DOM is difficult to predict as both increased and decreased Hg uptake in algae have been reported in the presence of DOM, depending on the algal species, DOM concentration and composition (Gorski et al., 2008; Le Faucheur et al., 2014; Luengen et al., 2012).

The aim of this study was to investigate the potential of transcriptomic to develop an early-warning biomarker tool of Hg-exposure in *Chlamydomonas reinhardtii* under environmentally relevant conditions. Previous analysis revealed that nM concentrations of IHg and MeHg are sublethal in *C. reinhardtii*, but induced an obvious and efficient defense response at the gene and cell level (Beauvais-Fluck et al., 2016, 2017). Briefly, both nM IHg and MeHg increased *chlorophyll a* content and increased photosynthesis efficiency, MeHg additionally increased intracellular reactive oxygen species (ROS) concentration and regulated a higher number of genes than IHg (Beauvais-Fluck et al., 2016, 2017). As toxic metals generally occur in mixtures in the aquatic environment, copper (Cu) was chosen to study its effect on Hg uptake because of i) its ubiquitous presence in freshwater, ii) its essentiality (*vs* nonessential Hg) to primary producers and iii) the previous observation of a competition between Cu and IHg uptake in a cyanobacteria and a macrophyte (Pandey and Singh, 1993; Regier et al., 2013b). Cu concentrations in aquatic systems have been reported from 0.4 to 400 µM, but it's known that its bioavailability and toxicity to organisms are highly dependent on its chemical speciation (USEPA, 2007; Zhang et al., 2017). We analyzed here the regulation of a subset of transcripts and linked transcript expression signatures to Hg intracellular concentrations, used as a direct measure of Hg bioavailability, in microalgae exposed to nM concentrations inorganic Hg (IHg) and/or methyl Hg (MeHg) in complex media including an essential metal (i.e. Cu) and humic substances (as proxy for the recalcitrant component of DOM). In addition the influence of IHg and MeHg on the gene expression triggered by Cu exposure was also considered.

2. Material and methods

2.1. Experimental design

Based on the global transcriptional analysis (RNA-Seq) of *Chlamydomonas reinhardtii* to IHg, MeHg and Cu in single exposure (Beauvais-Fluck et al., 2016, 2017), a subset of transcripts showing a specific response or a dose-dependent response to IHg, MeHg or Cu were selected. Suwannee River Humic Acid (SRHA) standard (International Humic Substances Society, St. Paul, MN, USA) was used as a proxy for the more recalcitrant fraction of DOM. We tested 0.7 or 70 nM IHg, 0.4 nM MeHg and IHg-MeHg mixtures (ratios IHg:MeHg of 1.75 or 175), in the presence or absence of 0.5 μM Cu and of SRHA (1 or 10 $mg{\cdot}L^{-1}$) to mimic conditions likely to be found in a Hg-contaminated site.

2.2. Labware

All material was washed in 10% HNO_3 (EMSURE, Merck, Darmstadt, Germany) followed by two 10% HCl acid baths (EMSURE, Merck, Darmstadt, Germany), thoroughly rinsed with ultrapure water (MilliQ Direct system, Merck, Darmstadt, Germany) and dried under a laminar flow hood. Material for culture and experiments, including media, were additionally autoclaved (1 bar, 121°C, 20 min) to avoid microbial contamination.

2.3. Exposure of algae

Chlamydomonas reinhardtii (wild type strain CPCC11, Canadian Phycological Culture Centre, Department of Biology, University of Waterloo, Waterloo, ON, Canada) were harvested in their mid-exponential growth phase and exposed in 100 mL of an artificial medium, containing $8.2{\cdot}10^{-4}$ M $CaCl_2$, $3.6{\cdot}10^{-4}$ M $MgSO_4$, $2.8{\cdot}10^{-4}$ M $NaHCO_3$, $1.0{\cdot}10^{-4}$ M KH_2PO_4 and $5.0{\cdot}10^{-6}$ M NH_4NO_3, pH was 6.9 ± 0.1. The cell density was $8.1 \pm 1.1{\cdot}10^5$ $cell{\cdot}mL^{-1}$. All exposures were conducted using three biological replicates. The exposure duration of 2 h was chosen based on previous data of Hg toxicokinetics and *in-situ* RNA-Seq that supported the interest of such length of exposure in the field because it allows targeting early-response genes that are more specific to the toxicant than latter gene response (Beauvais-Fluck et al., 2016; Dranguet et al., 2017a).

For uptake experiments, exposure medium was prepared and enriched (or not) with SRHA 24 h before the exposure experiment, while metals were added 30 min before algae. *C. reinhardtii* cells were exposed or not (control) 2 h to 0.7 or 70 nM IHg ($Hg(NO_3)_2$ standard solution, Sigma-Aldrich, Buchs, Switzerland), 0.4 nM MeHg (MeHgCl standard solution, Alfa Aesar, Ward Hill, MA, USA) or 0.5 µM Cu ($CuSO_4$ solution, Sigma-Aldrich, Buchs, Switzerland) and the following mixtures: 0.7 nM IHg + 0.4 nM MeHg, 70 nM IHg + 0.4 nM MeHg, 0.7 nM IHg + 0.5 µM Cu, 70 nM IHg + 0.5 µM Cu and 0.4 nM MeHg + 0.5 µM Cu, (without SRHA), 1 $mg \cdot L^{-1}$ SRHA or 10 $mg \cdot L^{-1}$ SRHA.

2.4. *Metal Uptake*

After exposure, cells (50 mL) were centrifuged (10 min, 1300*g*). Pellets of algae exposed to IHg or MeHg and Cu were resuspended in 1 mM ethylene·diamine·tetraacetic·acid (EDTA, Sigma-Aldrich, Buchs, Switzerland) + 1 mM cysteine (Sigma-Aldrich, Buchs, Switzerland) and 1 mM EDTA, respectively and centrifuged (10 min, 1300*g*). Both washing media were prepared with the metal-free exposure medium. This procedure eliminated metals loosely bound to cell walls to enable measure of intracellular metal concentration ($[\text{metal}]_{\text{intra}}$). Algal pellets were immediately freeze-dried (Beta 1-8 K, Christ, Germany).

Intracellular total Hg (THg = IHg + MeHg) concentration was determined on freeze-dried pellets by atomic absorption spectrometry using the Advanced Hg Analyzer AMA 254 (Altec s.r.l., Czech Republic). The detection limit (DL) defined as 3× the standard deviation (SD) of 10 blank measurements was 0.05 ng THg. The accuracy of the measurements was examined by certified reference material (CRM) MESS-3, showing 100 ± 0.1 % recovery. To measure Cu uptake, dry algal pellets were digested in 1 mL HNO_3 (Suprapur, Merck Darmstadt, Germany) at 90 ºC for 1 h and Cu concentration was measured by inductively coupled plasma mass spectrometry (ICP-MS; 7700x, Agilent Technologies, Morges, Switzerland) which DL was 0.18 $\mu g \cdot L^{-1}$ Cu.

Concentrations of THg, MeHg and Cu in media were determined by the MERX Automated Total Mercury Analytical System (Brooks Rand Instruments, Seattle, WA, USA), having a DL of 0.04 $ng \cdot L^{-1}$ THg, the MERX Automated Methyl Mercury Analytical System (Brooks Rand Instruments, Seattle, WA, USA) having a DL of 0.01 $ng \cdot L^{-1}$ MeHg and ICP-MS (see above), respectively. Effective concentrations in media for single and mixtures metal experiments were in average 0.36 ± 0.03 nM MeHg and 0.68 ± 0.02 nM THg for low concentration experiments, and 68.0 ± 1.8 nM THg in high concentration experiments. The concentration of Cu was in average 0.55 ± 0.004 µM Cu. The chemical speciation of IHg, MeHg, and Cu in media solutions were calculated from finally determined metal concentrations by an iterative procedure in Excel (Microsoft, Redmond, WA, USA), as described in (Beauvais-Fluck et al., 2018b).

2.5. Transcript response assessment by nCounter

The nCounter technology by NanoString Inc. (Seattle, WA, USA) (Geiss et al., 2008), a medium-throughput quantitative approach to study differential transcript expression, without the need to perform reverse transcription of mRNA to cDNA and subsequent polymerase chain reaction (PCR), was chosen to test transcript expression level as biomarker of metal exposure. A subset of transcripts was selected according to the correlation of their expression level with intracellular Hg or Cu concentrations in previous RNA-Seq experiment (Table S1) (Beauvais-Fluck et al., 2017), available in the Gene Expression Omnibus database (GSE65109). The set included 3 housekeeping transcripts (for input variation), 6 positive (for lane specific variation) and 8 negative (for background correction) internal controls. Total RNA was extracted from 50 mL of culture as previously described using TRI Reagent (Sigma-Aldrich, Buchs, Switzerland) (Beauvais-Fluck et al., 2016, 2017) and 500 ng RNA were used for nCounter analysis. After background correction and normalization, 5 transcripts were not further considered because of their too low signal, 192 transcripts passed quality controls (Table S1), including 122 transcripts not having and 70 transcripts having an annotation in the MapMan ontology (Thimm et al., 2004). Among the represented metabolic pathways, 9 transcripts were annotated to the ‘cell’ category (e.g. motility and development), 12 to the ‘transport’ category (including 3 metal transporters, e.g. zinc transport precursor, 1 ammonium and 3 ABC transporters), 7 to the ‘photosynthesis’ category (including 5 transcripts involved in the carbon concentrating mechanism), 7 to the ‘sugar metabolism’ category (glycolysis, TCA, major and minor) and 2 to the ‘tetrapyrrole synthesis’ and to the ‘oxidation-reduction’ (redox) categories. Other categories (e.g. hormone, secondary metabolism, lipid, nitrate and biodegradation of xenobiotics) were represented by 1 transcript each. The category ‘regulation of gene expression’ (i.e. RNA, protein, amino acid, nucleotide, signaling) included 23 transcripts and 3 transcripts were attributed to the ‘miscellaneous’ category in the codeset (Table S1).

2.6. Data analysis

Background THg and Cu concentrations in cells measured in the absence of SRHA and in presence of 1 mg·L^{-1} SRHA or 10 mg·L^{-1} SRHA were subtracted from data on metal uptake and t-tests (α=0.05) were used to compare metal uptake for each treatment *vs* Control. To compare the different treatments, uptake was normalized by the effective exposure concentration in media at the beginning of treatment ($[\text{metal}]_{\text{intra}}/[\text{metal}]_{\text{med}}$).

Fold changes (FC) for the nCounter analysis were calculated in Excel by comparing expression of transcripts in algae exposed to metal(s) (single and mixture), in absence or presence of 1 and 10 $mg \cdot L^{-1}$ SRHA and for algae exposed to SRHA alone *vs* control (absence of SRHA and absence of Hg, MeHg or Cu). This enabled to analyze both the molecular effects of metal and SRHA, and their interactions. Heatmaps were created in Genesis v1.7.7 (Institute for Genomics and Bioinformatics, Graz University of Technology, Graz, Austria) (Sturn et al., 2002).

Statistical analyses (t-tests, principal component analysis and histograms) and graphical representations were computed in Sigma Plot (Systat Software, San Jose, CA, USA).

3. Results

3.1. Uptake of IHg and MeHg in mixtures of metals

We assessed the Hg uptake by determining the intracellular metal concentrations and by comparing the ratios of intracellular THg concentrations to IHg and MeHg concentrations in the exposure medium (Figure 1A). While the presence of 0.5 µM Cu had no significant effect on THg uptake in mixtures with 0.7 nM IHg or with 0.4 nM MeHg, the THg uptake decreased five times when the concentration of IHg was increased to 70 nM (Figure 1A, Table S2). Importantly, the uptake of MeHg was much more efficient than uptake of Hg, as revealed by the ratio $[\text{THg}]_{\text{intra}}/[\text{THg}]_{\text{med}}$ being 19× higher for 0.4 nM MeHg than that for 0.7 nM IHg solutions.

The presence of SRHA had variable effects on THg uptake depending on metal composition (Figure 1B, Table S2). The addition of 1 $mg \cdot L^{-1}$ SRHA decreased THg uptake (for all exposure conditions except 70 nM IHg) as compared to systems with no humic substances. A similar intracellular concentration of THg was determined at 1 and 10 $mg \cdot L^{-1}$ SRHA for 0.7 nM IHg, 70 nM IHg + 0.4 nM MeHg and 70 nM IHg + 0.5 µM Cu. For 0.7 nM IHg + 0.5 µM Cu a significant decrease in THg uptake was observed when SRHA was increased from 1 to 10 $mg \cdot L^{-1}$ (t-tests, *p*-values<0.01) (Figure 1B, Table S2). To summarize, THg uptake in IHg treatments is decreased by SRHA in presence of Cu (0.7 and 70 nM IHg) or MeHg (70 nM IHg), while THg uptake is unaffected or even increased by SRHA for IHg treatments in absence of Cu and MeHg. In MeHg treatments THg uptake is generally not affected by Cu, IHg or SRHA. Although some of the results may be explained by uptake being controlled by chemical speciation and metal competition for uptake sites (Table S3), the general picture points at other mechanisms being of major importance for metal uptake in this organism.

3.2. Transcript signatures of IHg and MeHg alone and with SRHA

The expression level of the 192 transcripts strongly changed between the 27 experimental treatments (Table S1). A closer look at the $\log_2$FC values of IHg and MeHg in single exposure, revealed 25 transcripts showing contrasted regulation for 0.7, 70 nM IHg and 0.4 nM MeHg (Table 1). Transcript expression was also affected by the presence of SRHA and its concentration. For instance, $\log_2$FC values of the transcript Cre10.g447800, coding for an uncharacterized protein, decreased from 3.5 to 1.9 and 0.5 at 0.7 nM IHg with 0, 1 and 10 $mg \cdot L^{-1}$ SRHA, respectively. A transcript involved in signaling (Cre16.g668850) showed opposite regulation of $\log_2$FC for 0.7 nM (e.g. -4.0 at 0 $mg \cdot L^{-1}$ SRHA) and 70 nM IHg (e.g. 0.6 at 0 $mg \cdot L^{-1}$ SRHA). In line with the significant decrease in THg uptake for MeHg at 1 $mg \cdot L^{-1}$ SRHA, the expression of g18130 was 1.2× lower at 1 $mg \cdot L^{-1}$ SRHA. Furthermore, an amino acid transporter (Cre06.g298750) was up-regulated in 0.7 nM and 0.7 nM IHg + 1 $mg \cdot L^{-1}$ SRHA, and down-regulated in the other treatments (Table 1). This transcript could be an interesting candidate biomarker of IHg exposure in the nM range. Two transcripts, g6368 showing sequence similarity to the *Arabidopsis thaliana* MLO1 (putatively involved in the modulation of pathogen defense and leaf cell death) and g16833 (involved in post-translational modification), were specifically up-regulated by MeHg, and could thus be interesting candidate biomarkers of MeHg exposure. The expression of the 25 selected transcripts (Table 1) revealed that globally their expression level at 70 nM IHg was closer to 0.4 nM MeHg than to 0.7 nM IHg treatment, suggesting that single transcript responses could differentiate IHg exposure at the nM *vs* the μM range, but not IHg from MeHg in *C. reinhardtii*.

In the absence of SRHA, the expression of 23 and 15 transcripts (out of 25) was either unchanged or close to the arithmetical mean of their expression in the single IHg and MeHg treatments at 0.7 nM IHg + 0.4 nM MeHg and 70 nM IHg + 0.4 nM MeHg, respectively. At 0.7 nM IHg + 0.4 nM MeHg, 2 transcripts showed stronger regulation than at 0.7 nM IHg. At 70 nM IHg + 0.4 nM MeHg, the expression of 6 transcripts was stronger than in 70 nM IHg and 4 transcripts had an opposite regulation than in single IHg and MeHg treatments. Data suggested a higher and more specific transcript expression regulation by the interaction of IHg and MeHg when exposed in the 175 IHg:MeHg ratio treatment than in quasi-equimolar treatment.

The presence of SRHA had no effect on the transcript expression for 11 and 8 transcripts (4 were common) for 0.7 nM IHg + 0.4 nM MeHg and 70 nM IHg + 0.4 nM MeHg, respectively. For instance, the expression of Cre02.g109650, coding for a transcript involved in the cell motility, was always close to the arithmetical mean of single treatments, at all SRHA concentration tested here. On the opposite, the comparison of the expression of 3 and 4 transcripts to their expressions in single treatments differed in the three SRHA conditions, for 0.7 nM IHg + 0.4 nM MeHg and 70 nM IHg + 0.4 nM MeHg, respectively. These results suggest that SRHA had a significant impact on transcript expression, in single treatments but also in IHg-MeHg mixture, supporting that gene expression is a very sensitive variable.

3.3. Linking single transcript expression level and Hg intracellular concentrations

Here we aimed to assess the potential of developing a biomarker of Hg uptake based on transcripts' expression level. We thus selected transcripts showing the same $\log_2$FC signs among all IHg and MeHg treatments, resulting in a list of 11 candidate transcripts (Figure 2). Both FC and THg uptake were normalized by their values in absence of SRHA to compare all treatments, including mixtures with Cu and to account for the effect of SRHA (see above and Table S1). Only one transcript, g18130 (kinase) showed a decrease in transcript expression level with decreased uptake at 0.7 nM IHg, while the 10 other transcripts resulted in no obvious correlation, suggesting that this approach may not be very promising to predict Hg uptake in *C. reinhardtii*.

3.4. Linking multiple transcript expression signatures and Hg intracellular concentrations

Here, to assess the potential of transcript expression signature, we selected 60 transcripts for further investigation as biomarkers of metal uptake in all experimental treatments. Selection of transcripts was made on following criteria: i) transcripts showing a $\log_2$FC lower than -0.5 or higher than +0.5 in the '1 mg·L^{-1} SRHA' and '10 mg·L^{-1} SRHA' treatments were excluded to limit SRHA background signal; ii) the transcripts showing specific expression for 0.7 nM IHg, 70 nM IHg and MeHg and different expression level for mixtures with Cu were included, iii) transcripts that showed an altered expression with SRHA congruent with measured uptake were selected. Principal component analysis (Figure S1) showed that the signature of this subset of transcripts efficiently discriminated 0.7 nM IHg (and 0.7 nM + 0.4 nM MeHg) from 70 nM IHg (and 0.7 nM IHg + 0.4 nM MeHg), and to a lesser extent 0.4 nM MeHg from 70 nM IHg. The heatmap built with all the treatments showed similar outcomes as the principal component analysis (Figure 3, clusters A and C). Mixtures of IHg with Cu (cluster D) were clustered separately from Cu alone (0 and 1 mg·L^{-1} SRHA, cluster E) and IHg alone (clusters A and C).

The signature of the subset of transcripts also allowed classifying samples according to Hg uptake: e.g. 0.7 nM IHg + 10 mg·L^{-1} SRHA (0.003 ± 0.015 amol$_{\mathrm{THg}}$·cell^{-1}) was closer to the signatures of SRHA than to 0.7 nM IHg + 10 mg·L^{-1} SRHA (0.012 ± 0.015 amol·cell^{-1}). In cluster A, the samples classified according to measured [THg]$_{\mathrm{intra}}$, ranging from 17.2 to 9.5 amol$_{\mathrm{THg}}$·cell^{-1} from 70 nM IHg + 10 mg·L^{-1} SRHA to 70 nM + 0.4 nM MeHg + 10 mg·L^{-1} SRHA, respectively. We observed the same trend in cluster E, ranging from 2.47 amol$_{\mathrm{THg}}$·cell^{-1} to 0 amol$_{\mathrm{THg}}$·cell^{-1} (i.e. below background THg concentration in 10 mg·L^{-1} SRHA) from 70 nM IHg + 0.5 µM to 0.7 nM IHg + 0.5 µM + 10 mg·L^{-1} SRHA.

Five transcripts, that showed a strong down-regulation in Cu only treatment, were included in the subset of transcripts and successfully discriminated a specific signature for Cu treatments. The signature of transcripts exposed to Cu + 10 mg·L^{-1} SRHA was, however, close to the signature of SRHA, in line with the strong effect of SRHA on Cu uptake (see below).

As mentioned above, among the 192 transcripts studied by nCounter, we could identify few transcripts discriminating, 0.7 nM IHg, 70 nM IHg and 0.4 nM MeHg (Table 1), but their FC were not congruent with THg uptake (Figure 2). On the other hand, when plotting the distribution of log$_2$FC values for the 60 selected transcripts for all treatments including 1 and 10 mg·L^{-1} SRHA (Figures S2), we found the number of transcripts with high FC value to increase with increased intracellular concentration. We thus plotted the range of log$_2$FC values (difference between the lowest and the highest log$_2$FC values) and THg uptake normalized by the concentration of exposure ([THg]$_{\mathrm{intra}}$/[THg]$_{\mathrm{med}}$ ratio) for the 60 selected transcripts (Figure 4). A linear relationship was observed for 0.7 nM IHg and 0.7 nM IHg + 0.5 µM Cu (lower [THg]$_{\mathrm{intra}}$/[THg]$_{\mathrm{med}}$ ratio) (adjusted $R^2 = 0.84$) and another linear relationship for 70 nM IHg, 70 nM + 0.4 nM MeHg and 0.4 nM MeHg, in line with their higher [THg]$_{\mathrm{intra}}$/[THg]$_{\mathrm{med}}$ ratios (adjusted $R^2 = 0.92$) (Figure 4). Correlations were slightly weaker when 0.7 nM IHg + 0.4 nM MeHg or 0.4 nM MeHg + 0.5 µM Cu were included respectively with the lower [THg]$_{\mathrm{intra}}$/[THg]$_{\mathrm{med}}$ ratio (adjusted $R^2 = 0.58$). However, this linear correlation between transcript expression and Hg uptake appears to be promising for the further development of transcriptomic tools to assess Hg exposure and Hg bioavailability in mixtures.

3.5. Copper uptake and transcript expression in response to Cu exposure

For comparative purpose, we assessed the transcript expression and Cu uptake in complex media containing Cu. In the absence of SRHA, Cu intracellular concentration decreased in mixture with 70 nM IHg (2×) and 0.4 nM MeHg (1.7×) treatments, while it was unchanged in mixture with 0.7 nM IHg (Table S2). The presence of SRHA significantly decreased Cu uptake in all conditions (t-tests, p-values<0.01) (Table S2). More in detail, for the 0.5 µM Cu, Cu uptake decreased (1.9×) from 0 $mg \cdot L^{-1}$ SRHA to 1 $mg \cdot L^{-1}$ SRHA, but was unchanged from 1 to 10 $mg \cdot L^{-1}$ SRHA, while Cu intracellular concentration further decreased (2.2×) at 10 $mg \cdot L^{-1}$ SRHA for 0.7 nM IHg + 0.5 µM Cu. For both 70 nM IHg + 0.5 M Cu and 0.4 nM MeHg + 0.5 µM Cu treatments, measured intracellular concentrations in the presence of 10 $mg \cdot L^{-1}$ SRHA were below the background concentration of control cells exposed to 10 $mg \cdot L^{-1}$ SRHA (Table S2). These results imply that MeHg and SRHA have a strong negative effect on Cu uptake and that Cu in mixture with high IHg concentration affected both THg and Cu uptake in *C. reinhardtii*. The decrease of Cu uptake with increasing SRHA concentration was in fair agreement with a modeled decreased fraction of inorganic Cu, whereas negative impacts of Hg and MeHg on Cu uptake likely have little to do with competition given the large differences in concentrations (Tables S2 and S3).

Exposure to Hg, Cu and SRHA resulted in a wide range of Cu intracellular concentrations. For comparison, we plotted transcript FC values against Cu intracellular concentrations and found 7 transcripts showing dose-dependent response with Cu uptake in the studied mixtures (Figure S3). For instance, $\log_2$FC increased with increased uptake for the antioxidant enzyme glutathione peroxidase 5 (GPX5, Cre10g.458450), while $\log_2$FC decreased for Cre14.g615350 involved in oxygen transport. The Fe-assimilating protein (Cre12.g456600) showed a bell shape with increasing uptake of Cu. Data suggest that single transcript expression could assess more reliably Cu bioavailability than Hg bioavailability. However, both Hg bioavailability and Cu bioavailability were well correlated with transcript expression signature (see above).

4. Discussion

4.1. Impact of DOM on Hg and Cu uptake

We hypothesized that the presence of SRHA would decrease Hg, MeHg and Cu uptake and consequently impact the level of transcript regulation. In agreement with the above hypothesis, addition of SRHA decreased THg intracellular concentration in 0.4 nM MeHg treatments as well as Cu intracellular concentration in all treatments. The latter observation was consistent with the decrease of metal uptake in presence of DOM observed for many cations, e.g. Cu^{2+} and Cd^{2+}, and was attributed to the complexation of metals to DOM binding sites, such as oxygen-containing, amino and reduced sulfur functional groups, reducing the free metal ions concentrations and thus metal bioavailability (Lamelas and Slaveykova, 2007). It has been reported that DOC concentrations exceeding 100 μM decreased IHg and MeHg uptake in the diatom *Thalassiosira pseudonana* when exposed 1 h to natural waters spiked with 2 nM IHg or 0.9 nM MeHg (Zhong and Wang, 2009). Similarly, in the diatom *Cyclotella meneghiniana*, exposure during 72 h to 0.4-0.8 nM MeHg with increasing DOM concentration (0, 1.5, 3, 5, 10 and 20 mg·L^{-1} DOM isolated from natural waters) showed a decrease of MeHg uptake (Luengen et al., 2012). For the green alga *Selenastrum capricornutum*, additions of 10-20 mg·L^{-1} DOM decreased IHg and MeHg uptake after 24 h of exposure to 1 pM IHg and 3 pM MeHg (Gorski et al., 2008). A recent study further demonstrated that THg uptake in biofilms correlated with the predicted concentrations of IHg chemical species not bound to organic ligands in natural waters (Dranguet et al., 2017b). In contrast, THg uptake by *C. reinhardtii* in our study increased after addition of 10 mg·L^{-1} SRHA at 70 nM IHg. A doubling of MeHg uptake was reported in *C. reinhardtii* exposed to 0.6-0.7 nM MeHg with increased DOC (280 μM DOC *vs* 177 μM DOC) (Pickhardt and Fisher, 2007). Using a bacterial bioreporter it was observed that IHg bioavailability under non-equilibrium conditions significantly increased when the DOM concentration was increased from 0 to 10 mg·L^{-1} DOM, but the bioavailability declined upon further increase in DOM to 50 mg·L^{-1} (Chiasson-Gould et al., 2014). To summarize, despite many observations providing support for binding to DOM and competition with other metals being in control of IHg and MeHg uptake by organisms in natural waters, there are also results pointing at more complicated explanations. In this study the modeled chemical speciation of IHg, MeHg and Cu (dividing each metal into fractions involving organic and inorganic ligands; Table S3) is difficult to directly link with their bioavailability (Table S2), suggesting that other mechanisms are interfering.

We recently studied the impact on THg intracellular concentration of SRHA in the aquatic macrophyte *Elodea nuttallii* using similar experimental conditions as reported here (Beauvais-Fluck et al., 2018b). While addition of 1 mg·L^{-1} SRHA had no impact on uptake, an increase to 10 mg·L^{-1} SRHA significantly decreased THg intracellular concentrations in both IHg and MeHg 0.1 nM treatments. An increase of IHg to 10 nM had no further increasing effect on THg intracellular concentration. For both IHg + MeHg and IHg + Cu, addition of 10 mg·L^{-1} SRHA significantly reduced THg intracellular concentration (as compared to control and 1 mg L^{-1}), whereas for the MeHg + Cu mixture THg intracellular concentrations increased by

1.9× concomitantly with a 1.4× decrease in Cu intracellular concentrations. Based on speciation modeling, it was suggested that formation of Cu(I) in presence of *E. nuttallii* could explain these data together with the established difference in binding affinities for IHg and MeHg to DOM functional groups. These very clear differences in the uptake of IHg, MeHg and Cu between *E. nuttallii* and *C. reinhardtii* could be attributed to the unicellularity of the alga vs. the pluricellularity of the macrophyte. Another factor that differed between these two experiments is the surface-to-volume ratio which is expected to result in higher uptake in unicellular organisms (Beauvais-Fluck et al., 2018a). However, in contrast to this expectation, THg intracellular concentrations in *E. nuttallii* appeared to be higher than for *C. reinhardtii*, in line with field observations showing a high Hg uptake in *E. nuttallii* compared to other primary producers (Beauvais-Fluck et al., 2018a). Besides, based on transcriptome responses, the impact of IHg and MeHg uptake appears to be lower for *C. reinhardtii* than for *E. nuttallii* both in controlled and field experimental conditions (Beauvais-Fluck et al., 2018a; Dranguet et al., 2017a). Although *C. reinhardtii* harvests light via chloroplasts for energy as plants do, it also possesses numerous genes derived from the last plant-animal common ancestor that have been lost in angiosperms, including transporters and the possibility of extensive metabolic flexibility (Merchant et al., 2007). Taken together, our divergent observations on how an unicellular and a pluricellular organism take up IHg, MeHg and Cu may imply that homeostasis networks triggered by IHg, MeHg and Cu exposure are species-specific and modify the metal uptake by different organisms to an extent that chemical speciation modeling alone cannot explain.

4.2. Impact of Cu on Hg uptake and impact of Hg on Cu uptake

In aquatic ecosystems, toxicants are present in cocktails, thus to improve environmental realism of the exposures, here we further tested the impact of Cu on Hg uptake. We hypothesized that chemical mixtures will affect metal bioaccumulation, e.g. by direct competition for uptake or complexation with humic acid, or through synergistic interactions. We observed a 4.7× decrease of THg uptake in 70 nM IHg + 0.5 µM Cu, but no impact in 0.7 nM IHg + 0.5 µM Cu treatment. Concomitantly, we observed a 2x decreased Cu uptake in 70 nM IHg + 0.5 µM Cu treatment. In the cyanobacteria *Nostoc calcicola*, mixture exposure of 1.5 µM IHg + 40 µM Cu decreased 2× IHg uptake (Pandey and Singh, 1993). Here Cu competition is more effective when present in 10-20× excess than in higher excess. This could be attributed to Cu homeostasis network regulation that triggers different transporters according to external Cu concentration. This finding is different from previous observations made on the macrophyte *E. nuttallii* which was suggested to take up Hg using high affinity Cu transporters COPT1 (Regier et al., 2013a). Again, these contrasted results point to species-specific homeostasis networks triggered by IHg, MeHg and Cu exposure. Obviously, there is a need to gain fundamental knowledge on how various metals affect the bioavailability of other metals in different types of organisms.

4.3. Linking transcript expression level and uptake

A rapid diagnostic of environmental risk is desirable to limit and mitigate pollution impacts. Transcriptomic was reported to be more sensitive than classical bioassays (e.g. shorter exposure and low concentrations), and to have the potential of identifying the impact of several stressors in a single analysis (Dranguet et al., 2017a; Garcia-Reyero et al., 2009; Gomez-Sagasti et al., 2016; Regier et al., 2013a; Yang et al., 2007). It seems thus a promising approach in the context of toxicity and risk assessment but requires further testing in more complex experimental conditions. In this context, we aimed to investigate in detail the relationship between transcript expression level and exposure in more realistic environmental scenarios, using Hg uptake as a "proof of concept". Here, while it was possible to find 4 transcripts among the 192 transcripts discriminating 0.7 nM IHg, 70 nM IHg and 0.4 nM MeHg (Table 1), only 1 transcript had a regulation pattern significantly correlated in a dose-dependent manner to THg uptake (Figure 2). In the same line, two recent studies in aquatic primary producers showed that using single transcript expression is not sufficient as specific metal biomarker, while the expression signature of a subset of transcripts seems more promising. Simon *et al.* (2008, 2011) used single transcript expression as biomarker of Cd exposure in *C. reinhardtii*, first in controlled laboratory conditions and then *in situ* (Simon et al., 2011; Simon et al., 2008). None of the 9 potential biomarkers of Cd exposure in *C. reinhardtii* tested in binary metal mixtures with Cu or led by quantitative reverse transcription PCR confirmed their Cd specificity established in single exposure experiments (Hutchins et al., 2010). On the opposite, in *E. nuttallii* the signature of transcript expression measured by nCounter analysis after 24 h exposure in IHg + Cd mixture was able to differentiate mixture of 1 nM IHg + 1 nM Cu from 1 nM IHg + 0.1 nM MeHg and 1 nM IHg alone, confirming the sensitivity of this approach (Regier et al., 2013a).

Similarly, here measuring the signature of a subset of transcripts and the number of transcripts showing high FC values appeared to be a more consistent approach to assess Hg exposure. A strong correlation was obtained between THg uptake and the range of FC values, including for treatments resulting in low THg uptake such as in 0.7 nM IHg and complex media including Cu and SRHA. This is in line with recent transcriptomic studies on *C. reinhardtii* and *E. nuttallii* exposed 2 h *in situ*, where the number of transcripts with high FC (e.g. >|4|) was congruent with the gradient of contamination (up to 12 pM THg), although bioaccumulation of Hg was comparable with the background levels (Dranguet et al., 2017a). However, here the linear correlation was different for low and high $[THg]_{intra/}[THg]_{med}$ ratios in our experimental conditions, pointing that transcript expression showed the highest sensitivity at low Hg concentrations, which are more likely to be found in natural waters and difficult to assess by classical bioassays.

What is more, we observed here that the transcriptional profiling of the subset of 60 selected transcripts successfully clustered treatments according to the metal uptake in all experimental treatments including mixtures. Moreover, it is likely that the specific signatures are linked to the different mode of action of IHg, MeHg and Cu. The cluster of MeHg, close to 70 nM IHg, was consistent with a previous study on the whole transcriptome response to IHg and MeHg in *C. reinhardtii*, showing many common cellular pathways regulated by IHg and MeHg exposure, suggesting a common mode of action of both Hg forms (Beauvais-Fluck et al., 2017). Here, our results showed that MeHg and Cu have distinct modes of action as suggested by the opposite transcript expression signature observed.

For Cu, the intracellular Cu concentration was globally congruent with the modeled free Cu^{2+} concentration in the exposure medium (except in the most complex media; Tables S2 and S3) and congruent with the expression level of transcripts. On the opposite, data for Hg uptake were less straightforward than Cu data and confirmed the difficulty to use chemical modeling to predict Hg bioavailability even in simplified media in the presence of organic matter and other metals. Our data highlights the need of an accurate measurement of uptake that critically reflects bioavailability to cells. There is thus a need for novel tools, like transcriptomic, notably to assess Hg bioavailability for Hg risk assessment. Indeed, transcript expression signature could be an efficient biomarker of Hg and other contaminants exposure, because the expression of numerous transcripts depends on the interaction of the toxicant with intracellular biomolecules. Additionally, using transcript expression signature may be valuable in the context of risk assessment due to their high sensitivity and mechanistic value (Schirmer et al., 2010). In future research, the subset of transcripts needs improvement by adding or removing some transcripts as well as testing additional scenarios (e.g. a larger range of IHg, MeHg and DOM concentrations and their binary and ternary mixtures, several time points) as well as other toxicants and environmental samples. Notably, the effect on the gene expression level would need to be linked to effects at higher level of biological organization (i.e. individuals, population) to be applicable in risk assessment (Brinke and Buchinger, 2017). However, our data evidenced that using the expression signature of a subset of 60 transcripts was a promising tool to detect exposure to pollutants in *C. reinhardtii*. Notably, the range of FC values among regulated transcripts might represent a sensitive early-warning biomarker of exposure at low concentrations.

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Table 1: Fold changes ($\log_2$FC) of 25 selected transcripts showing differential expression for IHg and MeHg (--N.A.--, not assigned).

Transcript	MapMan category	0.7 nM IHg			70 nM IHg			0.4 nM MeHg		
		SRHA (mg·L^{-1})			SRHA (mg·L^{-1})			SRHA (mg·L^{-1})		
		0	1	10	0	1	10	0	1	10
Cre10.g447800	--N.A.--	3.48	1.95	0.54	-0.04	0.00	0.20	0.43	0.45	0.34
Cre06.g298750	transport-AA	2.92	1.39	-0.60	-0.37	-0.37	-0.68	-0.49	-0.73	-0.69
Cre02.g074800	--N.A.--	1.01	0.89	0.49	2.48	2.24	1.91	3.08	2.88	2.53
Cre02.g093500	--N.A.--	-0.14	0.03	-0.30	1.54	1.28	1.29	2.95	3.08	3.12
Cre12.g492650	--N.A.--	0.34	-0.34	-0.26	0.87	0.46	0.77	3.04	3.17	3.05
Cre06.g260550	nucleotide	0.92	0.47	-0.07	1.43	1.33	0.90	2.00	1.94	1.74
Cre16.g657200	xenobiotics	-0.10	0.37	0.68	0.78	0.41	0.91	1.22	1.18	1.53
g9144	cell motility	-2.39	-2.51	-2.19	-0.90	-1.21	-1.24	1.69	1.61	1.84
Cre16.g668850	signalling	-4.05	-3.26	-2.01	0.63	0.66	0.22	-0.11	0.11	-0.27
Cre17.g714300	--N.A.--	-3.26	-2.27	-1.69	0.24	0.13	-0.42	-0.09	0.04	-0.21
Cre14.g616050	--N.A.--	-2.39	-2.17	-1.44	0.58	0.40	0.05	0.20	0.35	0.07
Cre06.g249500	RNA	-2.19	-1.84	-1.38	0.69	0.41	-0.03	0.29	0.28	-0.06
Cre10.g442800	transport-misc	-0.69	-0.47	-0.33	-0.47	0.04	0.10	-0.16	-0.06	-0.11
Cre12.g529450	cell division	-1.03	-0.92	-0.79	-0.44	-0.49	-0.61	0.07	0.01	-0.03
Cre03.g152750	apoptosis	-0.72	-0.84	-0.86	0.16	-0.45	-0.25	0.23	0.50	0.57
g11558	--N.A.--	-0.80	-0.63	-0.93	0.84	0.36	0.20	0.63	0.49	0.20
Cre02.g109650	cell motility	-1.52	-1.00	-0.65	1.13	0.73	0.29	0.94	0.96	0.51
Cre06.g249350	RNA	-1.78	-1.23	-0.87	0.92	0.69	0.24	0.95	0.95	0.60
Cre10.g441250	--N.A.--	-0.67	-0.71	-0.36	-0.10	0.15	0.15	0.83	0.68	0.63
g6368	transport-misc	0.03	-0.16	-0.39	0.01	-0.09	-0.21	0.85	0.60	0.45
g16833	proteins	0.05	0.04	0.06	-0.08	-0.20	-0.20	0.46	0.40	0.54
Cre06.g263550	redox	-0.05	-0.24	-0.44	0.27	0.26	0.35	0.86	1.02	0.92
g18130	proteins	-0.14	-0.09	-0.13	0.46	0.46	0.40	1.09	0.94	0.99
Cre10.g458450	redox	-0.18	-0.21	-0.16	0.70	0.67	0.63	0.96	1.09	0.84
g6373	proteins	-0.21	-0.40	-0.55	0.68	0.74	0.58	1.12	0.77	0.86

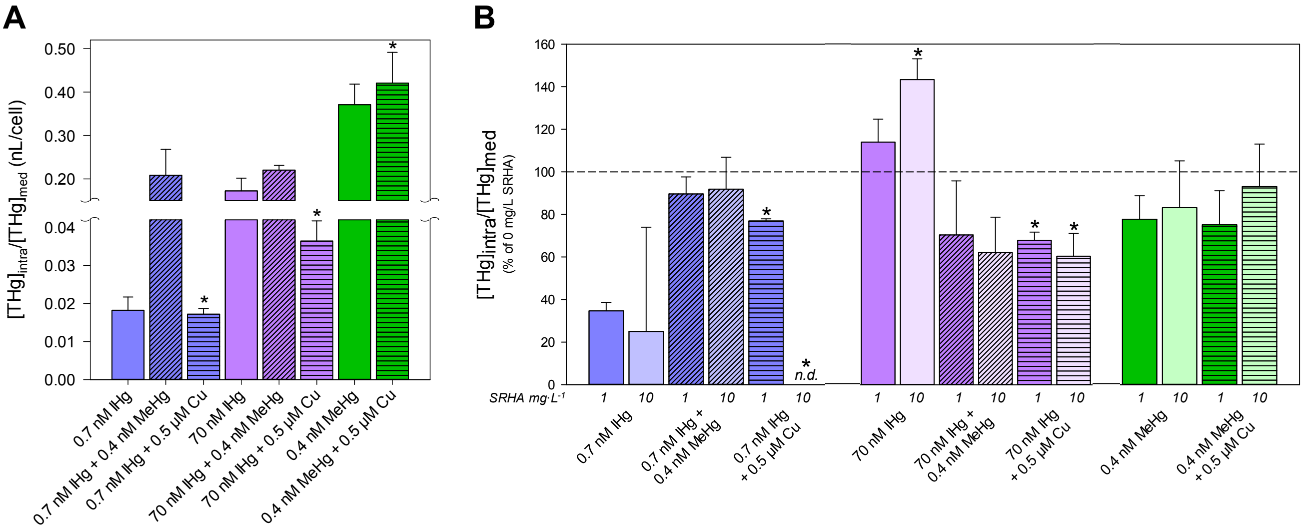

Figure 1: (A) Effect of MeHg on IHg uptake and effect of Cu on IHg and MeHg uptake in absence of SRHA. Asterisks indicate a significant difference with the respective treatment without Cu (t-test, *p*-value < 0.05). (B) Effect of SRHA on THg uptake for all treatments compared to the respective treatment without SRHA normalized by 100% (dashed line). Asteriks indicate a significant difference with the respective treatment without SRHA (ANOVA post-hoc Holm-Sidak, *p*-value < 0.05). Uptake was measured as THg (= IHg + MeHg) concentration in algal cells ($[\mathrm{THg}]_{\mathrm{intra}}$) and divided by concentration in the medium ($[\mathrm{THg}]_{\mathrm{med}}$) (mean ± SD, n = 3). Numbers 1 and 10 indicate concentration of SRHA ($\mathrm{mg \cdot L^{-1}}$).

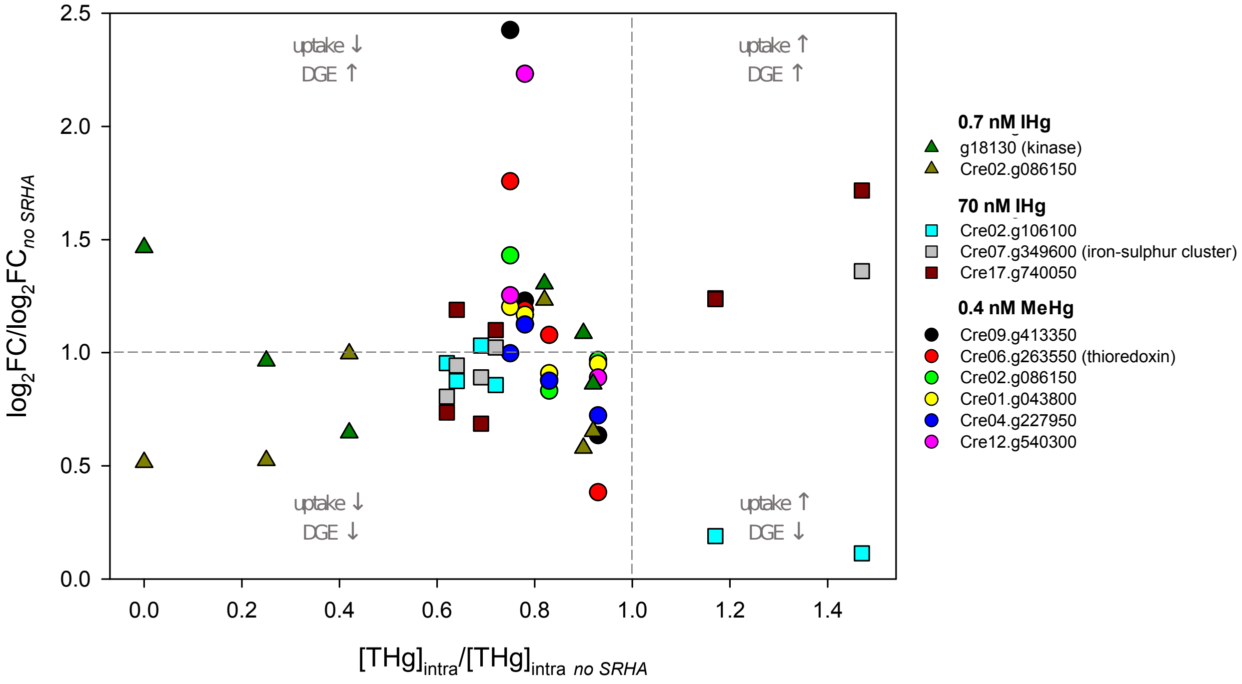

Figure 2: Gene fold changes for 11 selected genes according to THg uptake, both normalized by their value in absence of SRHA.

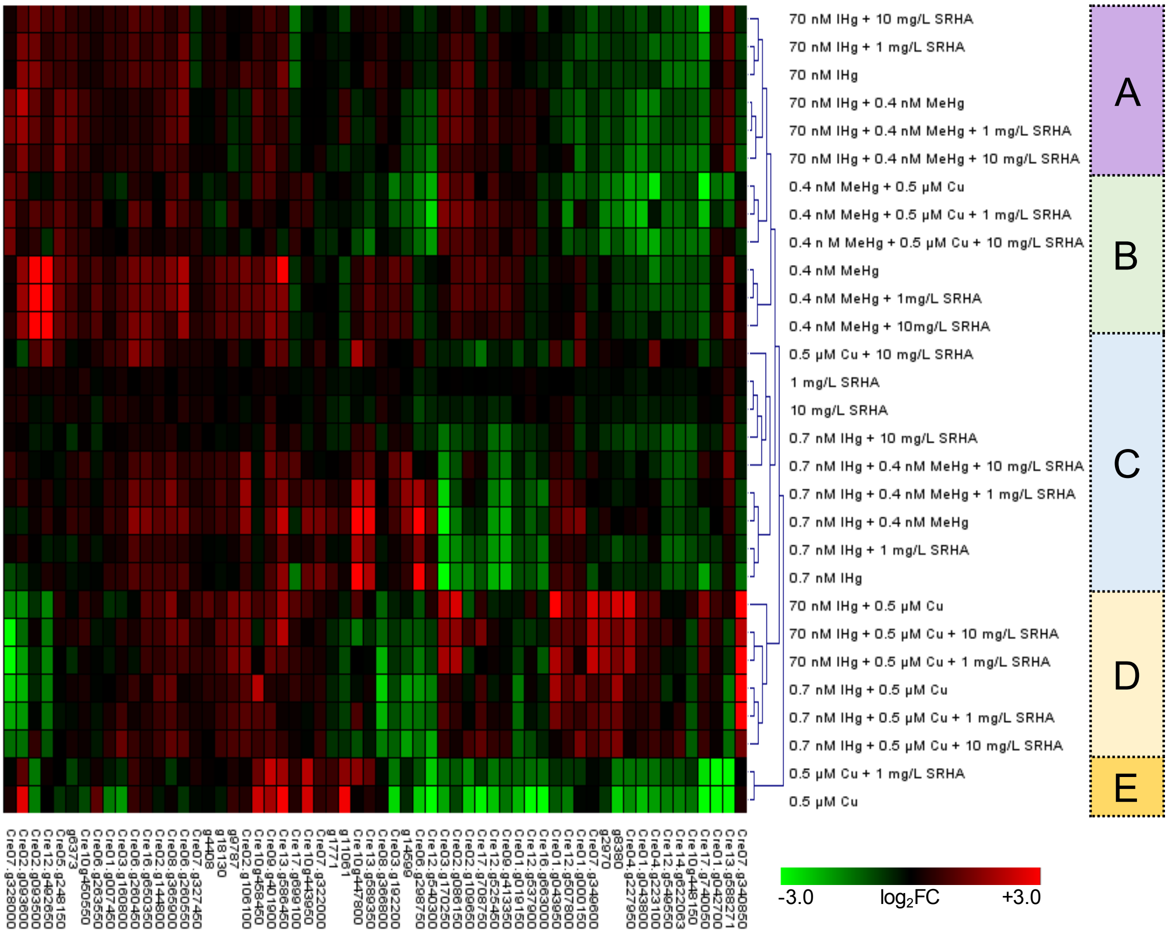

Figure 3: Hierarchical clustering of treatments according to the expression level of 60 selected genes (average linkage, Euclidean distance). Fold changes were calculated by dividing the expression level in the treatment by the expression level in the control (no metal, no SRHA). Letters show second level clusters.

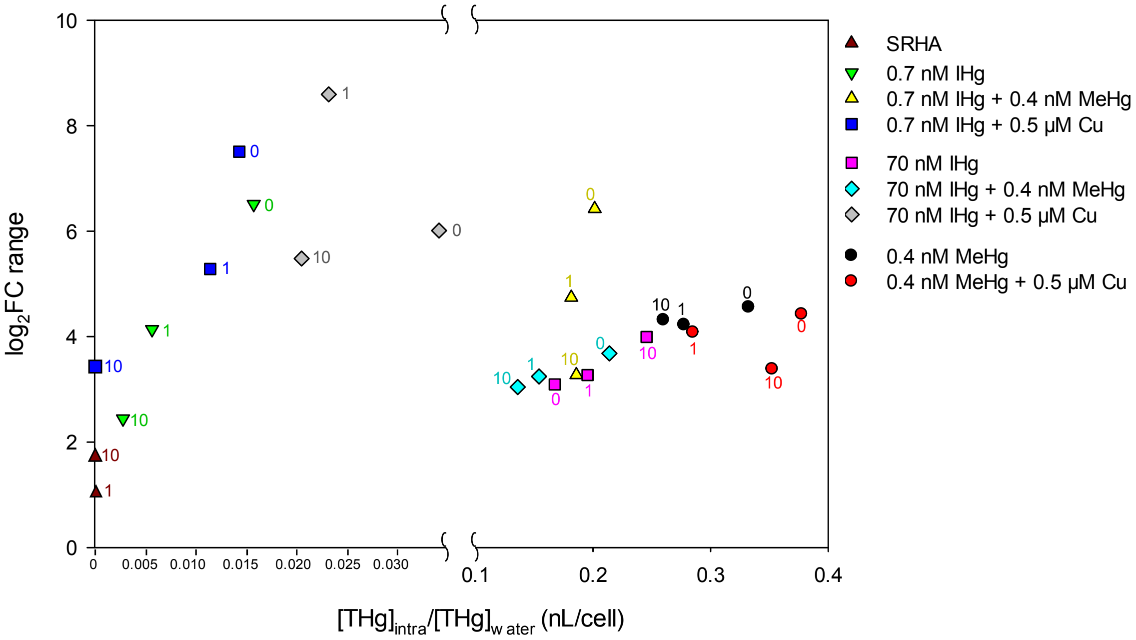

Figure 4: Relationship between the range of $\log_2$FC values for 60 selected genes (difference between the lowest and the highest $\log_2$FC values) and THg uptake normalized by medium concentration. Numbers 0, 1 and 10 indicate concentration of SRHA (mg·L^{-1}).