Role of cellular compartmentalization in the trophic transfer of mercury species in a **freshwater plant-crustacean food **chain****

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Abstract

Mercury (Hg) represents an important risk for human health through the food webs contamination. Macrophytes bioaccumulate Hg and play a role in Hg transfer to food webs in shallow aquatic ecosystems. Nevertheless, the compartmentalization of Hg within macrophytes, notably major accumulation in the cell wall and its impact on trophic transfer to primary consumers are overlooked.

The present work focusses on the trophic transfer of inorganic Hg (IHg) and monomethyl-Hg (MMHg) from the intracellular and cell wall compartments of the macrophyte *Elodea nuttallii* - considered a good candidate for phytoremediation - to the crustacean *Gammarus fossarum*.

The results demonstrated that Hg accumulated in both compartments was trophically bioavailable to gammarids. Besides IHg from both compartments were similarly transferred to *G. fossarum*, while for MMHg, uptake rates were ~2.5-fold higher in *G. fossarum* fed with the cell wall vs the intracellular compartment. During the depuration phase, Hg concentrations in *G. fossarum* varied insignificantly suggesting that both IHg and MMHg were strongly bound to biological ligands in the crustacean. Our data imply that cell walls have to be considered as an important source of Hg to consumers in freshwater food webs when developing procedures for enhancing aquatic environment protection during phytoremediation programs.

Keywords: cell wall, depuration, intracellular, toxicokinetics, bioaccumulation

1. Introduction

Mercury (Hg) is a pollutant known to bioaccumulate in organisms and that can cause serious threat to the ecosystems and eventually human health [1]. In the aquatic environment, Hg accumulation and effects have been widely studied in biota, notably in fish: the organic form of Hg, **monomethyl-Hg** (MMHg) and inorganic Hg (IHg) are both toxic, but MMHg has been shown to biomagnify through the food chain while IHg does not [2]. Dietary transfer of Hg is considered as being determinant for its effects on higher trophic animals [3]. Indeed, small increase of Hg levels in food can depress reproductive capabilities in herbivores, while direct exposure to similar concentrations is less detrimental [4]. Primary producers at the base of the food chain are thus **central** for the entry of Hg and its subsequent impact on higher trophic levels. Nonetheless, the precise factors controlling the transfer of Hg from primary producers to heterotroph consumers as well as the mechanisms and characteristics of these transfers remain poorly understood [5].

Macrophytes are key organisms of shallow aquatic environment in which MMHg is produced [6]. The ability of aquatic macrophytes to accumulate and tolerate high levels of IHg and MMHg **make them useful for** phytoremediation **purposes**, biomonitoring or for ecotoxicology **testing** [6-8]. On the other hand, **macrophytes** have been identified as having a role in Hg transfer to food webs in shallow aquatic ecosystems [2, 9]. Limited literature shows that accumulation of Hg in **plants and other** primary producers varies with species: **the flowering plant** *Elodea nuttallii* accumulated consistently higher Hg concentrations than **another flowering plant** *Ceratophyllum demersum* and seston sampled in several stations along a contaminated river in Romania [6, 10]. It was proposed that cell wall properties might explain in part differences in bioaccumulation in unicellular algae [11]. It is indeed well-known that plants use subcellular compartmentalization to limit the amount of toxic metal bound to sensitive targets [7, 12, 13]. Metals, including Hg, have been shown to be predominantly accumulated in cell walls in **macrophytic angiosperms exposed to** environmental concentrations **in the microcosm** and in the field [7, 14]. Besides, earlier **studies on other primary producers, in this case diatoms**, suggested that intracellularly accumulated MMHg was primarily transferred to copepods, while Hg accumulated in **membranes and silicate frustules** was not assimilated [15, 16], but this data awaits further confirmation in other primary producers to be generalized. In sum, the fate of Hg in cell wall *vs* internalized Hg, notably their respective transfer from macrophytes to herbivores and hence role in Hg transfer to food webs is **overlooked**.

The objective of this study was therefore to understand the role of IHg and MMHg compartmentalization in plants on the trophic transfer to invertebrates in an experimental **freshwater flowering** plant-invertebrate food chain. Recently we identified in **an** Hg-contaminated river in Romania, a macrophyte *E. nuttallii*, that showed high bioaccumulation of Hg, notably in its cell walls, and was at the base of the littoral-benthic food chain, representing hence a good model to study Hg transfer [2, 7, 8, 10, 17]. More in detail, we aimed to address the following questions: **(i)** Are IHg and MMHg accumulated in *E. nuttallii* transferred to *Gammarus fossarum* chosen as a surrogate for aquatic herbivores and decomposers? **and (ii)** How the compartmentation of Hg in the plant may influence its transfer to this primary consumer? This fundamental knowledge will help to further determine the susceptibility of Hg to enter **macrophyte-based** food web as well as will allow designing improved strategies for mitigation of Hg bioaccumulation and entry in food webs during phytoremediation programs.

2. Experimental

2.1 Labware **cleaning**

All material was soaked in (i) detergent 1 week, (ii) twice in HNO₃ 10% (v/v; *pro analysis* Sigma, Buchs, Switzerland) 1 week, (iii) in HCl 10% (v/v; *pro analysis* Sigma, Buchs, Switzerland) 1 week, thoroughly rinsed with MilliQ water (MilliQ Direct system, Merck Millipore, Darmstadt, Germany) after each washing step and dried under a laminar flow hood.

2.2. *Exposure of Elodea nuttallii*

Ten cm-long shoots of *E. nuttallii* without roots were collected **in the Etournels pound (France; 46°07'58"N and 05°56'46"E)**, brought to the laboratory and acclimated 48h in laboratory conditions: filtered water (1.2 µm) sampled in Lake Geneva, with controlled conditions at 20°C, 16/8h light/dark photoperiod, 1000 lux. Forty-eight shoots were exposed (or not: control) in 2.5L tanks for 2h to 0.2, 4, 20 µg·L⁻¹ MMHg (CH₃HgCl; Alfa Aesar, Ward Hill, MA, USA) or IHg (HgCl₂; Alfa Aesar, Ward Hill, MA, USA). The exposure concentrations in water were chosen based on previous studies to result in concentrations in shoots after 2h-long exposure covering the amount of Hg accumulated in macrophytes in the uncontaminated to contaminated environments [2, 6].

To assess the impact on compartmentalization in plants on subsequent Hg transfer, shoots were processed by two different procedures applied to retain Hg accumulated either

intracellularly or in cell walls while conserving the shoot morphology. To prepare shoots containing only intracellular Hg, shoots were rinsed for 10min with ethylene-diamine-tetra-acetic acid (EDTA; 1 mM; AppliChem, Darmstadt, Deutschland) and cysteine (1 mM; Sigma-Aldrich, Buchs, Switzerland). This extraction procedure is based on the competition for Hg binding between ligands on biota and molecules with high affinity to Hg and was previously shown to be the highly efficient for removal of IHg and MMHg in the cell wall [7]. To prepare shoots containing only Hg bound in the cell wall, shoots were soaked 3d in methanol-chloroform (v/v 2:1; Sigma, Buchs, Switzerland) to remove membrane phospholipids and hence dissolve membranes and purify intact cell walls as described before [7]. Shoots were in addition rinsed for 5min in milliQ water prior and after both treatments.

2.3. Exposure of *Gammarus fossarum*

Gammarus fossarum were collected from the Bourbre River (France) from the same population as in [18, 19]. Organism sampling was carried three weeks before exposure, allowing acclimatization in laboratory conditions (drilled groundwater, water temperature 12°C, constant oxygenation, conductivity $\sim 500 \mu\text{S}\cdot\text{cm}^{-1}$, 16/8h light/dark photoperiod, *ad libitum* feeding with alder leaves. Alder leaves were analysed for their Hg ($0.04 \pm 0.02 \mu\text{g}\cdot\text{total}\cdot\text{Hg}\cdot\text{g}^{-1}$ d.w.) and MMHg ($0.95 \pm 0.02 \text{ ng}\cdot\text{MMHg}\cdot\text{g}^{-1}$ d.w.) background concentrations.

As operated in previous study [19], we took care in the selection of exposed organisms to limit any confounding influence of biotic factors on the level of Hg in tissues. In that way, nine individual males homogeneous in size (1 cm-long), were exposed in triplicates in 500 mL polypropylene beakers with oxygenated water maintained at a temperature of 14°C and renewed continuously ($8 \text{ renewals}\cdot\text{d}^{-1}$) to limit direct Hg exposure by the water (e.g. aqueous released Hg from plants). Ten shoots were added to the beaker at the beginning of the exposure. Two exposure scenarios were conducted: Experiment 1 (bioaccumulation) consisted in a 7 day-long exposure of gammarids to plants pre-exposed to the different concentrations of IHg and MMHg. This experiment allowed to assess the importance of Hg transfer from macrophytes to gammarids and subsequently to choose an optimal concentration for experiment 2. Experiment 2 (toxicokinetics) consisted in 2, 4, 7 day-long exposure of gammarids to plants exposed to the intermediate concentrations of IHg and MMHg and 7 days of further depuration to assess dynamics of transfer according to Hg form and shoot's compartment. For depuration, individuals were transferred to a clean beaker and fed 7

supplemental days with alder leaves. At the end of the experiments, gammarids were collected, carefully dried and immediately snap frozen in liquid nitrogen.

2.4. Determination of mercury in water, macrophytes and gammarids

Total Hg (THg = MMHg + IHg) concentration in freeze-dried organisms was measured using an AMA-254 (Altec s.r.l., Dvůr Králové nad Labem, Czech Republic). The detection limit was $0.003 \mu\text{g}\cdot\text{THg}\cdot\text{g}^{-1}$. The accuracy of the measurements was checked by analysing the certified reference material (CRM) MESS-3. The measurement error was usually about 3% and always below 6%.

THg concentration in water was measured with a MERX (Brooks Rand instruments, Seattle, WA, USA) following THg procedure [20]. The detection limit was $0.03 \text{ ng}\cdot\text{THg}\cdot\text{L}^{-1}$. The accuracy of THg measurements was tested by analyzing the CRM ORMS-5. The measurement error was usually about 2% and always below 5%.

MMHg in freeze-dried and ground gammarids was extracted by HNO_3 (Suprapur®, Merck Darmstadt, Germany) leaching, 12h at 60°C . Organisms and water samples were then analyzed with a MERX (Brooks Rand instruments, Seattle, WA, USA) following MMHg procedure [21, 22]. The detection limit was $0.002 \text{ ng}\cdot\text{MMHg}\cdot\text{L}^{-1}$. The accuracy of MMHg measurements was tested by analyzing the CRM TORT-2. The measurement error was usually about 15% and always below 25%.

Concentrations in water were measured at the beginning of the exposure of plants (Table A1). For IHg exposure scenarios, MMHg was below the detection limit, and hence $\text{THg} = \text{IHg}$. For MMHg, we considered that $\text{THg} = \text{MMHg}$ in water as no demethylation is expected in water in these conditions as shown previously [7].

2.5. Modelling of Hg accumulation by *Gammarus fossarum*

The variation of concentrations in gammarids with time can be modelled by a toxicokinetic one-compartment equation, allowing uptake and excretion rates to be estimated [23]. Our data highlighted the absence of significant excretion over the 7-day-long exposure period considered (data not shown), hence a simplified linear model to describe accumulation kinetics over time was used (Eq.(1)):

$$C_m(t) = C_m(0) + a \times t \quad \text{Eq.(1)}$$

where $C_m(t)$ is the Hg concentrations in gammarids ($\mu\text{g}\cdot\text{g}^{-1}$ dw) at time t (days); $C_{m(0)}$ is the constitutive Hg concentration measured in gammarids at the beginning of the experiment and a is the uptake rate ($\mu\text{g}\cdot\text{Hg}\cdot\text{g}_{\text{gammarid}}^{-1}\cdot\text{d}^{-1}$). The uptake rate a depends on both the concentration of Hg species in *E. nuttallii* compartments (C_{plant} , in $\mu\text{g}\cdot\text{Hg}\cdot\text{g}_{\text{plant}}^{-1}$) and an uptake rate constant (k_u , in $\text{g}_{\text{plant}}\cdot\text{g}_{\text{gammarid}}^{-1}\cdot\text{d}^{-1}$) representing the exposure of gammarids to contaminated plants, linked to assimilation efficiency (AE; **conversion of resource biomass into consumer biomass**) and ingestion rate (IR; **biomass of resource ingested per day**). This uptake rate constant can be derived according to the equation (Eq.(2)):

$$k_u = \frac{a}{C_{\text{plant}}} \quad \text{Eq.(2)}$$

2.6. Statistical analysis

We performed an analysis of variance (ANOVA, $p < 0.05$) with a post-hoc Tukey's HSD test ($p < 0.05$) for pairwise comparisons of bioaccumulation data. The uptake rates were estimated by fitting the toxicokinetic model with a linear model function (lm) [24]. When residuals were skewed, variance functions (power and exponential) were applied and the best model was selected according to Akaike's Information Criterion (AIC, pgirmess R package). Differences in parameter estimates between exposure modalities and subcellular fractions were checked using a generalized likelihood ratio test (GLRT). All statistics were performed with the free statistical software package R version 3.1.3 [25].

3. Results and discussion

3.1 Hg accumulation and compartmentalization in *E. nuttallii*

Accumulation of THg and MMHg in **both** the intracellular and the cell wall compartment of *E. nuttallii* was **first determined** (Table 1). The obtained THg and MMHg **contents** in shoots covered the whole range of concentrations found in *E. nuttallii* collected in clean ($0.05 \mu\text{g}\cdot\text{THg}\cdot\text{g}^{-1}$) **and in** contaminated sites, that reached for example $2 \mu\text{g}\cdot\text{THg}\cdot\text{g}^{-1}$ in a Hg-contaminated reservoir in Romania [2, 10]. As *E. nuttallii* is highly tolerant to Hg, toxicity of Hg to this plant, here as well as in natural water, is very unlikely [7, 10].

Mercury bioaccumulation in *E. nuttallii* after 2h at the lowest concentrations of exposure ($0.2 \mu\text{g}\cdot\text{L}^{-1}$) was insignificant compared with non-exposed shoots (Control) for both THg and MMHg and intracellular compartments (Table 1). In terms of concentrations, bioaccumulation of THg is higher for the cell wall than the intracellular compartment, in particular for MMHg

20 $\mu\text{g}\cdot\text{L}^{-1}$ exposure treatment. These findings are in line with previous studies showing ~60% of THg being bound in cell walls in *E. nuttallii* after 4 days of exposure at 76 $\text{ng}\cdot\text{L}^{-1}$ IHg and 23 $\text{ng}\cdot\text{L}^{-1}$ MMHg in water [7], hence supporting an important role of the cell wall in Hg binding. Indeed, cell walls of primary producers are very important sink for Hg [7, 14]. Moreover, differences in Hg accumulation in algae were attributed to their cell wall properties and proposed to control intracellular concentrations and hence the transfer in consumers [11]. On the other hand, comparable bioaccumulation of MMHg than IHg in both the intracellular and the cell wall compartments after 2h (Table 1) suggests similar accumulation capacity for both species at the studied concentrations. This could be attributed to the short-term exposure. For comparison, in *E. nuttallii* steady-state internal concentration of 0.40 and 1.09 $\mu\text{g}\cdot\text{THg}\cdot\text{g}^{-1}$ was reached after more than 8h for 23 $\text{ng}\cdot\text{L}^{-1}$ MMHg and 48 h for 76 $\text{ng}\cdot\text{L}^{-1}$ IHg, respectively [7]. Nevertheless, another study with *Chlamydomonas reinhardtii* exposed 48h to IHg and MMHg under low environmentally relevant concentrations in the laboratory in absence of organic matter, also reported similar intracellular concentrations for IHg and MMHg, supporting similar accumulation capacities for IHg and MMHg in this green alga, a close relative of flowering plants, in line with their similar lipid solubility [16, 26]. On the opposite, a 2- to 4-fold higher uptake of MMHg than IHg is regularly reported in natural food webs [2, 6, 15, 16]. These discrepancies mostly highlight that mechanisms resulting in biomagnification of MMHg, but no biomagnification of IHg in food webs are not understood in detail yet.

Concerning MMHg, in our conditions background THg concentration measured in unexposed shoots (Control) consisted of ~80% and 100% MMHg in the intracellular and the cell wall compartment, respectively (Table 1). The ratio of MMHg to the THg decreased for shoots exposed to IHg and increased both in intracellular and cell wall compartments exposed to MMHg compared to control conditions (Table 1). Here, no demethylation for MMHg exposure (i.e. MMHg = THg), and no methylation is expected for IHg exposure in water in this short-term exposure conditions as observed in previous experiments [7]. Besides, our data support the absence of substantial demethylation by shoots during bioaccumulation as indicated by $\geq 100\%$ MMHg/THg proportion in the cell wall and intracellular compartments, except in the intracellular compartment for MMHg 0.2 $\mu\text{g}\cdot\text{L}^{-1}$ resulting in 0.57 ± 0.29 MMHg/THg ratio. At this latter concentration, a demethylation was observed in shoots of *E. nuttallii*. Similarly, in phytoplankton and zooplankton a minor demethylation and negligible methylation have been reported after 48h- and 2 week- exposure, respectively, to IHg and MMHg [26, 27].

3.2 Hg accumulation in *Gammarus fossarum*

3.2.1 Role of Hg compartmentalization in *E. nuttallii*

Experiment 1 consisted in feeding gammarids with the intracellular or the cell wall compartments of *E. nuttallii* pre-exposed to various IHg and MMHg concentrations and measuring Hg bioaccumulation in gammarids after 7 days. To exclude confounding or indirect effects, the mortality was monitored daily and dead animals were removed. Cannibalism was thus avoided, which ensured that gammarids were only exposed to Hg through plants consumption. The mortality of gammarids during exposure was around 15% in all conditions. This weak mortality was not correlated with Hg treatments (concentration or IHg/MMHg), nor with the plant treatment (intracellular/cell wall) highlighting the absence of toxicity of our experimental conditions.

Concentrations measured in gammarids fed with control intracellular or cell wall compartments were in the range previously found for amphipods collected in the field ranging from 0.05 to 0.2 $\mu\text{g}\cdot\text{THg}\cdot\text{g}^{-1}$ [19, 28, 29]. THg and MMHg concentrations both linearly increased in gammarids with the concentrations found in *E. nuttallii* compartments (Figures 1 and 2), suggesting that assimilation efficiency (AE) and ingestion rate (IR) were unaffected by the range of Hg concentration tested here. On the other hand, the cell wall compartment resulted in higher slopes and concentrations in gammarids than the intracellular compartments (Figure 1A), suggesting that THg in the cell wall was more transferable to gammarids than THg in the intracellular compartment. Nevertheless, it can be questioned whether AE and IR were similar amongst the gammarids fed with the intracellular compartment and the cell wall compartment, as we were unsuccessful in estimating food amount ingested by gammarids. Previous studies concerning IHg and MMHg showed that AE were different for killifish (*Fundulus heteroclitus*) fed with different food sources, e.g. amphipods or worms [5]. Besides, here when considering THg, the higher slope of the linear increase of THg in *G. fossarum* with THg in *E. nuttallii* suggested that MMHg was more transferable than IHg (Figure 1A). In the same line, the ratio of MMHg to THg increased with increasing MMHg exposure from 0.14 ± 0.05 to 0.78 ± 0.05 and 0.16 ± 0.02 to 0.47 ± 0.09 for MMHg 0.2 and 20 $\mu\text{g}\cdot\text{L}^{-1}$ in intracellular and cell wall compartments, respectively. Nevertheless, MMHg to THg ratios in gammarids are globally lower than MMHg/THg ratio in shoots, suggesting that a significant part of MMHg is demethylated during feeding in our conditions, while no methylation is observed in the IHg exposure conditions. The above findings are consistent with the observation that most of the initial MMHg being demethylated in seston (70%) and

zooplankton (13%) in their experimental food chain involving algae and zooplankton [27]. Moreover, when looking at IHg and MMHg concentration in gammarids after feeding with MMHg-exposed-shoots vs concentration in shoots, IHg resulted in a higher slope than MMHg (Figure 1B), which is opposite to expected results based on MMHg biomagnifications in natural food chains [2, 6]. Indeed, AE of MMHg has been repeatedly shown to be higher than AE of IHg [5, 30-33]. Unfortunately, most studies only measured THg, hence suffering from a lack of reliable information concerning AE for MMHg and IHg and not allowing comparison with our data. However, here we can question whether AE was comparable for IHg and MMHg as both Hg form shows different binding sites at the subcellular level in shoots and might subsequently show different trophic availability [10, 33]. It was proposed that in plants, Hg could be retained in cell walls by means of extracellular carbohydrates, such as pectic sites or histidyl groups forming very stable complexes, or be associated with thiols or cysteines of cell wall proteins like extensin, which contains a Cys-rich domain [34]. Similarly, the intracellular Hg is expected to be bound via a sulfhydryl cysteine group of biothiols such as glutathione or phytochelatins and exported to vacuoles [35, 36]. In the intracellular fraction of different fish preys, MMHg was proposed to be bound in higher proportion to heat-stable-protein than IHg and thus more available for trophic transfer to fish [33]. Here, it is not known if IHg and MMHg are really bound in a similar manner in shoots of *E. nuttallii* and if their availability for the trophic transfer is similar. However, linear correlation between Hg in gammarids and shoots supported that Hg in the cell wall and in the intracellular compartment in the form of MMHg and IHg were all trophically available, at least in part, to gammarids.

3.2.2 *Kinetics of Hg transfer from shoots compartments to G. fossarum*

To gain further insights on Hg transfer dynamics in *G. fossarum*, we determined the THg, IHg and MMHg toxicokinetics in organisms exposed to moderately contaminated *E. nuttallii*, i.e. IHg $4 \mu\text{g}\cdot\text{L}^{-1}$ (Figure 2) and MMHg $4 \mu\text{g}\cdot\text{L}^{-1}$ (Figure 3), resulting in between 0.4 to 0.8 $\mu\text{g}\cdot\text{THg}\cdot\text{g}^{-1}$ in shoots' compartments (Table 1) for 7 days and further 7 days of depuration. Generally, the one-compartment model without taking into consideration the excretion adequately described the Hg accumulation from *E. nuttallii* to gammarids as reflected by the coefficients of determination (R^2 ; Table 2).

In *G. fossarum* exposed to IHg contaminated *E. nuttallii*, the exposure compartment (intracellular or cell wall) did not influence the accumulation patterns, i.e. no significant bioaccumulation of MMHg (although it represented 7 to 10% of the THg in plants, Table 1),

and a linear increase of bioaccumulated IHg vs time (0.020 and $0.023 \mu\text{g}\cdot\text{IHg}\cdot\text{g}_{\text{gammarid}}^{-1}\cdot\text{d}^{-1}$ for intracellular and cell wall compartments, respectively; Table 2). Considering the similar concentration of THg and IHg in the two types of diet, we calculated uptake rate constants of 30 and $31 \text{ g}_{\text{plant}}\cdot\text{g}_{\text{gammarid}}^{-1}\cdot\text{d}^{-1}$ (Table 2). Hence, the type of diet did not seem to influence the exposure of gammarids: IHg appeared to be similarly trophically transferred from the intracellular and the cell wall compartment of *E. nuttallii* to *G. fossarum*.

For MMHg exposed organisms, we evidenced important differences in the Hg availability and transfer according to the exposure compartment with uptake rates ~ 2.5 -fold higher in *G. fossarum* exposed to the cell wall compartment compared with the intracellular compartment (Table 2). To account for differences in Hg concentrations in the intracellular and cell wall compartments, uptake rate constants (k_u) were derived and revealed different values than those calculated for IHg exposure, i.e. higher for THg (0.051 and $0.067 \text{ g}_{\text{plant}}\cdot\text{g}_{\text{gammarid}}^{-1}\cdot\text{d}^{-1}$) and lower for MMHg (0.014 and $0.021 \text{ g}_{\text{plant}}\cdot\text{g}_{\text{gammarid}}^{-1}\cdot\text{d}^{-1}$; Table 2). In addition to confounding effects possibly due to different (i) AE and IR and (ii) subcellular distribution of IHg and MMHg discussed above, two hypotheses may be further evoked. Firstly, the gammarids fed differently according to the Hg species in the food. However, this kind of behavioural avoidance is unlikely because of the relatively low concentrations measured in the *E. nuttallii* compartments (from 0.4 to $0.8 \mu\text{g}\cdot\text{THg}\cdot\text{g}^{-1}$). Secondly, the gammarids had access to less MMHg than expected due to demethylation. This second hypothesis is supported by two results: (i) the decrease of MMHg/THg ratios with time in *G. fossarum* tissues (Figure 3) and (ii) the significant and quite elevated uptake rates modelled for IHg (Table 2) while this Hg species is absent from *E. nuttallii* compartments at the beginning of exposure (Table 1). The demethylation was more pronounced for the cell wall than the intracellular compartment, notably for MMHg $20 \mu\text{g}\cdot\text{L}^{-1}$ ($p < 0.05$). However, it remains unclear when this demethylation occurred, i.e. in the plant tissues on which the gammarids fed (due to, for instance, plant material decay and microbial biotransformation), or within the gammarids (during digestion, metabolization).

During the 7 day-long depuration phase, and in line with the linear accumulation patterns, Hg concentrations in *G. fossarum* did not significantly vary (Figures 2 and 3). Data therefore suggest that in gammarids the accumulated IHg and MMHg were strongly bound to biological ligands, e.g. proteins (sulfhydryl groups) or small peptides such as glutathione or Hg-cysteine complexes [35-37]. Recently, it was found that about 90% of Hg was accumulated in the cytosolic fraction of *Chironomus riparius* midge larvae exposed to IHg spiked sediments and partitioned between the sensitive (mostly enzyme) and detoxified (metallothionein-like)

components [23]. This latter kind of detoxification strategy probably also could explain the absence of excretion in *G. fossarum* in our exposure condition [38]. Indeed, Experiment 1 (bioaccumulation, Figure 1) showed that internal concentrations may reach much higher levels, without significant toxicity, than those found during Experiment 2 (kinetic; Table 2), when exposed to higher Hg concentrations. Gammarids were therefore certainly able to progressively sequester all the IHg and MMHg entering their tissues during Experiment 2 preventing both the induction of toxicity and the excretion of Hg.

To summarize, *G. fossarum* fed on *E. nuttallii* accumulates IHg and MMHg from both intracellular and cell wall compartments. In line with the existing literature, when measuring THg, MMHg-exposed shoots leads to a higher accumulation of THg than IHg-exposed shoots, but when measuring MMHg, we observed a significant demethylation of MMHg in gammarids compared to plants. However, here in our experimental conditions, we did observe a significant biotransfer of IHg and MMHg. Moreover, our data evidenced that opposite to the general hypothesis, Hg bound in the cell wall of flowering plants such as *E. nuttallii* can be transferred and assimilated by *G. fossarum* as is Hg found in the intracellular compartment. The previous finding that mostly intracellular MMHg was transferable to primary consumers was conducted on diatoms, which cell wall made of silica (hydrated silicon dioxide; frustule) is completely different from pecto-cellulosic cell wall of plants, and copepods [16]. Moreover, opposite to copepods, gammarids used in the present study feed on leaves and thus are able to degrade cell walls of plants. In the same line, it was found that the amphipods *Hyallela azteca* had higher capacity to assimilate Hg, than copepods feeding on a deteriorated diatom culture [15]. Besides, it was demonstrated that trophic transfer of MMHg from the phytoplankton in the water column was the major uptake route for the estuarine amphipod *Leptocheirus plumulosus* [39], suggesting that our findings are generalizable to other photosynthetic organisms, notably plants and microalgae.

Although the subcellular metal distribution is determinant for many consumers that are unable to assimilate the insoluble fraction of the cell, the digestive strategies to handle food of the consumer are also important. Hence, feeding behaviour of consumers might be central for the fate of Hg in food webs. Therefore, species abundance, notably their capacities of bioaccumulation and feeding respectively for primary producers and consumers, may be considered for enhancing aquatic environment protection as they will significantly influence the fate of Hg in food webs during phytoremediation programs.

4. Conclusion

The plant-animal interface plays a crucial ecological and ecotoxicological role in aquatic ecosystems as a driver of nutrients and contaminants to higher trophic levels. The present study on accumulation of IHg and MMHg in the macrophyte *E. nuttallii* and then dietary transfer to macroinvertebrates demonstrated that Hg species in both the intracellular and the cell wall compartments are significant sources of Hg for the macroinvertebrate *G. fossarum*. As cell wall constituents are recalcitrant by nature, they are expected to remain longer in the environment. Our data hence point out the cell wall as a new important source of Hg transfer to consumers in local food webs to be considered for enhancing aquatic environment protection during phytoremediation programs. Moreover, our data supports that the different binding sites at the subcellular level in shoots of MMHg and IHg might subsequently result in different trophic availability to consumers. Besides, our data suggest that different AE for IHg and for MMHg in consumers might explain the biomagnification of MMHg in food webs.

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Figure legends

Figure 1: Hg concentration ($\mu\text{g}\cdot\text{g}^{-1}$) in *G. fossarum* as a function of Hg concentrations ($\mu\text{g}\cdot\text{g}^{-1}$) in plants at the beginning of gammarids exposure. (A) THg concentration in *G. fossarum* fed 7 day-long with the IHg or MMHg contaminated intracellular or cell wall compartment of *E. nuttallii* as a function of THg concentrations. (B) IHg or MMHg concentration in *G. fossarum* fed 7 day-long with the MMHg-contaminated intracellular or cell wall compartment of *E. nuttallii* as a function of respectively the IHg and MMHg concentrations in plants. Slopes and R^2 of the respective linear regressions are indicated. Values with different letters are significantly different ($p<0.05$; $n = 3$).

Figure 2: THg, MMHg and IHg accumulation in *G. fossarum* fed with the IHg contaminated intracellular (A) or cell wall (B) compartment. The vertical line represents the beginning of the depuration phase.

Figure 3: THg, MMHg and IHg uptake in *G. fossarum* fed with the MMHg contaminated intracellular (A) or cell wall (B) compartment. The vertical line shows the beginning of the depuration phase.

Figure 1

Figure 2

Figure 3

Table 1: THg and MMHg concentrations measured (mean $\pm$ SD) in shoots of *E. nuttallii* ($\mu\text{g}\cdot\text{g}^{-1}$) at the beginning of exposure of *G. fossarum*. Values that share similar letters are not significantly different (ANOVA with the post-hoc Tukey's HSD test, $p<0.05$; $n = 3$).

Exposure treatment	THg		MMHg	
	Intracellular	Cell wall	Intracellular	Cell wall
Control	0.05 $\pm$ 0.01 a	0.04 $\pm$ 0.001 a	0.04 $\pm$ 0.02 a	0.07 $\pm$ 0.02 a
0.2 $\mu\text{g}\cdot\text{L}^{-1}$MMHg	0.07 $\pm$ 0.004 a	0.11 $\pm$ 0.004 a	0.04 $\pm$ 0.02 a	0.11 $\pm$ 0.05 a
4 $\mu\text{g}\cdot\text{L}^{-1}$MMHg	0.41 $\pm$ 0.02 b	0.76 $\pm$ 0.07 b	0.51 $\pm$ 0.21 b	0.76 $\pm$ 0.11 b
20 $\mu\text{g}\cdot\text{L}^{-1}$MMHg	4.18 $\pm$ 0.48 e	6.98 $\pm$ 0.52 c	7.06 $\pm$ 1.32 c	8.22 $\pm$ 4.60 c
0.2 $\mu\text{g}\cdot\text{L}^{-1}$IHg	0.06 $\pm$ 0.01 a	0.09 $\pm$ 0.01 a	0.05 $\pm$ 0.04 a	0.04 $\pm$ 0.02 a
4 $\mu\text{g}\cdot\text{L}^{-1}$IHg	0.71 $\pm$ 0.17 b	0.83 $\pm$ 0.07 b	0.05 $\pm$ 0.09 a	0.09 $\pm$ 0.04 a
20 $\mu\text{g}\cdot\text{L}^{-1}$IHg	7.15 $\pm$ 0.26 c	8.67 $\pm$ 1.27 c	0.07 $\pm$ 0.02 a	0.04 $\pm$ 0.02 a

Table 2 Toxicokinetic parameters (uptake rate a , uptake rate constant k_u) of THg, IHg and MMHg accumulation in *G. fossarum* fed with the MMHg or IHg contaminated intracellular or cell wall compartment of *E. nuttallii*. For each Hg form, values that share similar letters are not significantly different (glrt; ns = not significant; nd=not determinable).

Hg species	Exposure	$a \pm \text{SE}$ $\mu\text{g}\cdot\text{Hg}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$	k_u $\text{g}_{\text{plant}}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$	R ²
IHg				
THg	intracellular	0.020 ± 0.001^a	0.028	0.90
	cell wall	0.023 ± 0.002^b	0.028	0.89
MMHg	intracellular	$-0.0002 \pm 0.0001^{\text{ns}}$	nd	0.79
	cell wall	$0.0001 \pm 0.0001^{\text{ns}}$	nd	0.49
IHg	intracellular	0.020 ± 0.001^a	0.030	0.90
	cell wall	0.023 ± 0.001^b	0.031	0.90
MMHg				
THg	intracellular	0.021 ± 0.003^a	0.051	0.80
	cell wall	0.051 ± 0.009^b	0.067	0.56
MMHg	intracellular	0.007 ± 0.001^c	0.014	0.66
	cell wall	0.016 ± 0.002^d	0.021	0.70
IHg	intracellular	0.014 ± 0.003^c	nd	0.67
	cell wall	0.036 ± 0.009^d	nd	0.38