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## Subcellular Distribution of Dietary Methyl-Mercury in *Gammarus fossarum* and Its Impact on the Amphipod Proteome

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1 **Subcellular distribution of dietary methyl-mercury in *Gammarus***  
2 ***fossarum* and its impact on the amphipod proteome**

3

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21

22 **Abstract**

23 The transfer of methyl-Hg (MeHg) from food is central for its effects in aquatic animals, but  
24 we still lack knowledge concerning its impact in invertebrate primary consumers. In aquatic  
25 environments, cell walls of plants are particularly recalcitrant to degradation and as such  
26 remain available as food source for long periods. Here, the impact at the proteomic level of  
27 dietary MeHg in *Gammarus fossarum* was established and linked to subcellular distribution of  
28 Hg.

29 Individuals of *G. fossarum* were fed with MeHg in cell wall or intracellular compartments of  
30 *Elodea nuttallii*. Hg concentrations in subcellular fractions were 2 to 6x higher in animals fed  
31 with cell wall than intracellular compartments. At the higher concentrations tested, the  
32 proportion of Hg in metal sensitive fraction increased from  $30.0\pm 6.1\%$  to  $41.0\pm 5.7\%$  for  
33 individuals fed with intracellular compartment, while biologically detoxified metals fraction  
34 increased from  $30.0\pm 6.1\%$  to  $50.0\pm 2.8\%$  when fed with cell wall compartment. Data  
35 suggested that several thresholds of proteomic response are triggered by increased  
36 bioaccumulation in each subcellular fraction in correlation with Hg exclusively bound to the  
37 metal sensitive fraction, while the increase of biologically detoxified metal likely had a cost  
38 for fitness. Proteomics analysis supported that the different binding sites and speciation in  
39 shoots subsequently resulted in different fate and cellular toxicity pathways to consumers.

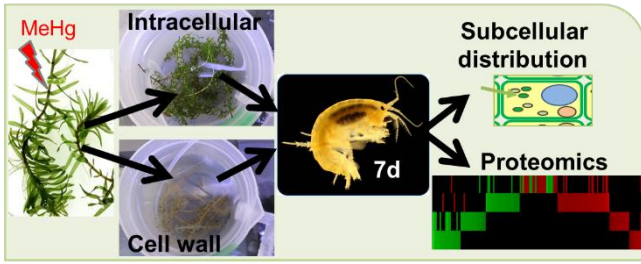
40 Our data confirmed that Hg bound in cell walls of plants can be assimilated by *G. fossarum*,  
41 which is consistent with its feeding strategy, hence pointing cell walls as a significant source  
42 for Hg transfers and toxicity in primary consumers. The high accumulation of Hg in  
43 macrophytes make them a risk for food web transfer in shallow ecosystems. The present  
44 results allowed gaining new insights on the effects and uptake mechanisms of MeHg in  
45 aquatic primary consumers.

46  
47 Keywords: bioaccumulation; cell walls; cytosol; proteogenomics; subcellular distribution.

48  
49 Synopsis: subcellular compartmentalization of MeHg in shoots determines its fate and toxicity  
50 in primary consumers

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54

## 55 **Introduction**

56 Mercury (Hg) is a global pollutant seriously threatening ecosystems and eventually health,  
57 due to the biomagnification of methyl-Hg (MeHg) through the food chain <sup>1, 2, 3</sup>. Past Hg  
58 deposits of anthropogenic sources continue to cycle through compartments, forming up to  
59 60% of current Hg emissions <sup>4</sup>. Consequently, even if new emissions of Hg are kept constant,  
60 Hg deposition will increase in the future <sup>5, 6, 7</sup>. In this context, understanding how Hg is  
61 bioaccumulated and biomagnified in food webs is central to increase the effectiveness of  
62 environmental management programs for biota and human health. For example, aquatic plants  
63 are key species in shallow aquatic environment in which MeHg is formed <sup>8</sup>. The high  
64 accumulation of Hg in aquatic plants make them considered a model species for  
65 phytoremediation programs, biomonitoring or to perform ecotoxicological tests <sup>9</sup>, but also at  
66 risk for food web transfer <sup>10, 11</sup>. A current research priority is to establish the sources of dietary  
67 Hg having a detrimental impact in aquatic plant-based food webs for mitigation programs.

68 Both inorganic Hg (IHg) and MeHg are bioaccumulated in biota. While MeHg generally  
69 represents 0.05 to 1% and up to 30% of total Hg (THg = IHg + MeHg) in water, MeHg forms  
70 up to 80-100% of the THg in fish muscles, resulting in very high toxic body burdens in fish,  
71 therefore threatening humans' health through fish consumption <sup>2, 12, 13</sup>. Consequently,  
72 although both IHg and MeHg trigger toxicity in biota, in animals dietary MeHg is the main  
73 risk of Hg <sup>2, 12</sup>. Nonetheless, a major gap in knowledge concerns the impact of dietary Hg  
74 uptake in invertebrate primary consumers. Data supported the oxidative stress to be central for  
75 MeHg toxicity in aquatic animals <sup>14, 15</sup>, but few studies concern effects through diet at  
76 environmental concentrations and none concern invertebrates <sup>16</sup>. In this context, the impact of  
77 MeHg in realistic environmental exposure scenarios and representative biota need to be  
78 studied to better anticipate its potential environmental impact. In aquatic invertebrates,  
79 toxicity and tolerance of metal accumulation can be explained by its subcellular partitioning  
80 <sup>17</sup>. For example, in biota the sequestration of toxic metal by metallothioneins and granules is  
81 known as biologically detoxified metals (BDM), while the partition in the metal sensitive  
82 fraction (MSF) is expected to cause the toxicity <sup>17, 18</sup>. Moreover, linking intracellular  
83 concentration and distribution to molecular and cellular responses is a current research  
84 priority in biomonitoring, as it could participate in revealing innovative biomarkers for  
85 environmental risk assessment <sup>9, 19</sup>.

86 Pioneer studies on diatoms suggested that intracellular MeHg was primarily transferred to  
87 copepods, while Hg from cell walls, consisting in silicate frustules, was not assimilated <sup>20</sup>. By  
88 contrast, we recently demonstrated that IHg and MeHg from cell walls -formed primarily of

89 cellulose, hemicellulose and pectin- in shoots of the macrophyte *Elodea nuttallii* resulted in a  
90 higher transfer to gammarids than intracellular Hg<sup>10</sup>. Similarly, chironomids fed with  
91 chopped macrophytes, to mimic incorporation in sediments of decaying tissues, revealed that  
92 MeHg was more transferable to sensitive subcellular targets and bioavailable fraction in  
93 primary consumers than IHg found in intracellular or cell wall compartments<sup>11</sup>. Data point  
94 that the composition of cell walls could affect the transfer rate of Hg to consumers<sup>10, 11, 21</sup>.  
95 The proportion of diatoms in phytoplankton is particularly high in nutrient-rich marine coastal  
96 and high latitudes ecosystems, but significantly less in freshwater rivers, lakes and other  
97 environments<sup>22, 23, 24</sup>. In this context, data concerning Hg food transfer in marine plankton  
98 might not be generalized to other animals with distinct feeding strategies or other ecosystems,  
99 including freshwater, brackish water or contaminated sites. Moreover, studies on effect of  
100 trophic Hg exposure in primary consumers, i.e. invertebrates are rare<sup>10, 11, 21</sup>. At  
101 environmental concentrations, cell walls appeared as the main bioaccumulation compartment  
102 of Hg in macrophytes and because of their low degradability are expected to remain for long  
103 periods in the aquatic systems, likely serving as food source<sup>25, 26, 27</sup>. Here, we hypothesized  
104 that the binding of Hg to plant's cell walls results in a higher dietary transfer and subsequently  
105 in a higher toxicity in primary consumers than Hg bound in plant's intracellular compartment.  
106 Gammarids as generalist herbivores are instrumental in European lotic ecosystems food webs  
107 because of their role in the breakdown of the leaf litter. They are also good bioaccumulators  
108 of metals and good candidates for ecotoxicological testing of freshwater ecosystems<sup>19, 28</sup>.  
109 *Gammarus* spp are found in *E. nuttallii* beds and were previously reported to feed on the latter  
110<sup>29, 30</sup>. Here we aimed to address how the MeHg localization in *E. nuttallii* shoots (cell wall vs  
111 intracellular) defines its subcellular distribution in *G. fossarum*; and how dietary MeHg  
112 impacts the proteome of *G. fossarum*. Gammarids were fed for 7 days with either cell wall or  
113 intracellular compartment of the plant *E. nuttallii* previously exposed to MeHg and we  
114 analyzed the fate and effects of MeHg in animals through subcellular distribution and shotgun  
115 proteomics. Extensive proteome profiles further revealed the molecular toxicity pathway of  
116 dietary MeHg. This fundamental knowledge contributes to a better knowledge of the fate and  
117 impact of dietary MeHg in food webs.

118

## 119 **Material and Methods**

### 120 *Labware cleaning*

121 Glasswares and plastic consumables for exposures and sampling were washed for once in  
122 detergent, twice in 10% HNO<sub>3</sub>, once in 10% HCl, rinsed after each washing step with MilliQ

123 water (Merck Millipore) and kept in a laminar flow hood until dry. Glass material was in  
124 addition heated overnight at 200°C.

125

#### 126 *Elodea nuttallii* and *Gammarus fossarum* exposure

127 Exposure protocols of *E. nuttallii* and *G. fossarum* were described in <sup>10</sup>. Briefly, shoots were  
128 collected in Etournels ponds and acclimated 48h in laboratory conditions. Shoots (10 cm)  
129 without roots were exposed to MeHg (CH<sub>3</sub>HgCl; Alfa Aesar) or not exposed (control). The  
130 exposure concentrations in water were chosen based on previous studies to result in  
131 concentrations in shoots after 2h-long exposure spanning lowly to highly contaminated  
132 environments (Table S1) <sup>2, 31</sup>. After the exposure, shoots were washed to conserve the shoot  
133 morphology and retain Hg either from intracellular or cell wall compartment. Briefly,  
134 intracellular Hg was conserved in shoots by rinsing with 10<sup>-3</sup> M EDTA (AppliChem) and 10<sup>-3</sup>  
135 M cysteine (Sigma-Aldrich) to remove the extracellular loosely bound Hg<sup>8</sup>. Hg bound in the  
136 cell wall were obtained by soaking shoots in methanol-chloroform 3d (Sigma), resulting in  
137 lipids solubilization and consequently allowing to recover an intact insoluble cell wall matrix  
138 with a conserved structure <sup>32</sup>. This latter method affects cell integrity and organic solvents  
139 could cause IHg and MeHg redistribution. As such, concentrations might not be strictly  
140 representative of initial concentrations in shoots but was performed to allow feeding animals  
141 with purified cell walls.

142 *Gammarus fossarum* were sampled in the Bourbre River (France) <sup>33, 34</sup>, and acclimatized three  
143 weeks to laboratory conditions. Nine 1 cm-long males were fed during 7 days with *E. nuttallii*  
144 intracellular (I) or the cell wall (CW) compartments pre-exposed to 4 and 20 µg L<sup>-1</sup> MeHg  
145 concentrations, thereafter referred as ‘low’ and ‘high’ MeHg treatments in line with their  
146 concentrations corresponding to lowly and highly contaminated environments, respectively <sup>2</sup>  
147 <sup>10, 11, 31</sup>. To ensure exclusive exposure of gammarids through plant consumption, water  
148 renewal and daily removal of dead individuals was done to avoid exposure to Hg through  
149 water or cannibalism, respectively. We observed a weak (15%) mortality of gammarids  
150 during exposure in all conditions <sup>10</sup>. This rate agrees with the criteria from the Afnor standard  
151 (XP T 90-722-3) for feeding rate measurement in the sentinel species *G. fossarum* <sup>35</sup>. It was  
152 not correlated with MeHg concentration, or with the plant compartment (intracellular/cell  
153 wall) and supported an absence or a sublethal toxicity in these experimental conditions <sup>10</sup>. No  
154 depuration was applied here, based on a former study, using the same experimental exposure  
155 conditions showed that a 7 day-long depuration didn’t varied Hg concentrations <sup>10</sup>.

156

157 *Subcellular fractionation of Hg in Gammarus fossarum*

158 The fractionation procedure of the whole body (Figure 1) adapted from <sup>17, 36</sup> was described  
159 elsewhere <sup>37</sup>. Analyses were done in triplicates. Two frozen gammarids per conditions were  
160 homogenized in 1 mL of ice-cold buffer (0.02M Tris-HCl pH 7.5; Sigma-Aldrich, purity  
161 99%). The homogenate (H) was centrifuged 30 min at 4°C at 10,000g. The pellet (P1) was  
162 hydrolyzed at 100°C 2 min and 1h with 1M NaOH 65°C and centrifuged 10 min at 10°C at  
163 10,000g, resulting in a supernatant including gut content, cellular debris and likely part of  
164 mitochondria (S2), while the pellet contained metal-rich granules and exoskeleton (P2). The  
165 supernatant (S1) was ultracentrifuged 30 min at 4°C at 100,000 g forming a pellet (P3)  
166 including lysosomes and microsomes and a supernatant (S3) with the cytosol. However, no  
167 marker enzymes (i.e. <sup>38, 39</sup>) was used to confirm further the nature of each subcellular fraction  
168 besides operational fractions according to <sup>17</sup>. All S3 was further filtered with a sterivex filter  
169 (millipore) at 0.45 µm, and thereafter named S4.

170 High-performance liquid chromatography (HPLC) was performed to fractionate S4 with a  
171 column using steric exclusion allowing (BIOSEP-SEC-S 2000; 1 - 300 kDa; Figure S1).  
172 Eluting fractions of 0.5 mL were collected up 18 mL, acidified at 0.5% with suprapur HCl  
173 (Merx) and kept at 4°C until analyzed. Three pools of metal-ligand were separated: (i) high-  
174 molecular weight (HMW; 20-255 kDa) eluting 9 to 15 min after injection, (ii)  
175 metallothionein-like protein eluting 16 to 24 min after injection (MTLPs-MW; 1.8-20 -kDa)  
176 and (iii) low-molecular weight (LMW; <1.8 kDa) eluting 25 to 35 min after injection. Using  
177 this protocol, MTLPs-MW are biologically detoxified metal in cytosol, while the heat  
178 sensitive protein (HSeP) or proteins in cytosol is the sum of HMW and LMW <sup>36</sup>. We also  
179 summed the area of peaks at 280 nm in these three fractions and normalized them by the  
180 biomass used in H. We eventually compared the sum of Hg amounts found in the different  
181 subcellular fractions with the fraction H, using  $H = P2 + S2 + P3 + HMW + MMW + LMW$  as  
182 a quality control, and observed a good agreement ( $n = 18: 93.4 \pm 4.9\%$ ). We also injected  
183 periodically 1 mM EDTA using the same mobile phase to wash the HPLC column and to  
184 verify that Hg concentrations in eluting fractions remained at background level. For the  
185 biological interpretation, metal sensitive fraction (MSF) was calculated as  $P3 + HSeP$  and  
186 biologically detoxified metals (BDM) as  $P2 + MTLPs-MW$ . As such,  $MSF + BDM + S2 = H$ .



187

188 *Determination of Hg in gammarids*

189 Pellets, i.e. P2 and P3 fractions, were resuspended in 1 mL water and mineralized 12h at 60°C  
190 30% HNO<sub>3</sub> (suprapur, Merck). THg concentration in P2, S2, P3, S4, MTPLs and HSeP were  
191 measured with a MERX-T analytical system (Brooks Rand instrumentsA) using THg method  
192 <sup>40</sup> showing a limit of detection (DL) of 0.03 ng·THg·L<sup>-1</sup>, an error of 2% using certified  
193 reference materials (CRMs) ORMS-5 (95.0 ± 1.7 % recovery) and TORT-2 (100 ± 0.1%  
194 recovery). MeHg was analyzed in P2, S2, P3, S4, MTPLs and HSeP samples with a MERX-  
195 M (Brooks Rand instruments) using MeHg method <sup>41,42</sup> showing a DL of 0.002 ng·MeHg·L<sup>-1</sup>,  
196 an error of 15% using the CRM TORT-2 (102.5 ± 2.4 % recovery).

197 Data are presented as arithmetic means ± standard deviations (SD). Normality and  
198 homoscedasticity were tested. An analysis of variance (ANOVA,  $p < 0.05$ ) with a post-hoc  
199 Tukey's HSD test ( $p < 0.05$ ) was used to perform pairwise comparisons of bioaccumulation in  
200 the software R version 3.1.3 <sup>43</sup>.

201

202 *Protein extraction and Tandem mass spectrometry*

203 Proteins were extracted (n= 6) from individuals of *G. fossarum* and identified following the  
204 same protocol as formerly described <sup>44</sup>. Briefly, each animal was subjected to a bead beating  
205 homogenization using a single 3.2 mm steel bead per tube in LDS buffer (20 µl·mg<sup>-1</sup>;  
206 Invitrogen). Cellular debris were eliminated after centrifugation for 3 min at 10,000 g.  
207 Proteins from the supernatant were denatured for 5 min at 99 °C. An aliquot of 20 µL was  
208 then loaded onto a SDS-PAGE gel for a brief (5 min) electrophoresis as recommended <sup>45</sup>.  
209 Each proteome sample was processed and proteolyzed with trypsin (Roche) in ProteaseMAX  
210 surfactant (0.01% , Promega) as recommended <sup>46</sup>. Peptides were analyzed with a Q-Exactive  
211 HF mass spectrometer (Thermo) operated in data-dependent mode with the same parameters  
212 as previously described <sup>47</sup>. Peptides were resolved at a flow rate of 0.2 µL·min<sup>-1</sup> along a  
213 90 min gradient of CH<sub>3</sub>CN in presence of 0.1% formic acid.

214

215 *Peptide identification and proteomic data analysis*

216 MS/MS spectra were interpreted against the GFOSS database, that is available through  
217 PRIDE (accession PXD024583 as GFOSS\_db01.fasta) with the MASCOT software (Matrix  
218 Science) and the same parameters as those previously described <sup>48</sup>. Proteins were validated on  
219 the basis of at least two identified peptides, resulting in a false discovery rate (FDR) lower  
220 than 1% as estimated with the decoy database search option. Abundance of each peptide was

221 measured by their Spectral Count (SC), which corresponds to the count of MS/MS spectra  
222 attributed to the same peptide. The protein abundance is the sum of the SC of all the peptides  
223 assigned to the protein. Protein abundances were compared between organisms fed with  
224 control plants or plants pre-exposed to MeHg. Abundance of Methionine and Cysteine residue  
225 content in proteins from each sample was calculated by extracting spectral counts of these  
226 residues as previously proposed <sup>49</sup>, *i.e.* multiplying the spectral counts of each protein by its  
227 number of S-containing residues. The weighted relative abundance in S-containing residues  
228 was then obtained by dividing the sum of S-spectral counts by the sum of spectral counts of  
229 all residues.

230 Proteomics data have been deposited to the ProteomeXchange Consortium via PRIDE <sup>50</sup> with  
231 the dataset identifier PXD024583 and project DOI 10.6019/ PXD024583

232

### 233 *Gene Ontology (GO term) functional annotation, classification and analysis*

234 Identified proteins were annotated in April 2018 using Swissprot (<https://www.uniprot.org/>)  
235 and NCBIInr (<https://www.ncbi.nlm.nih.gov/protein/>) as databases explored with Diamond, a  
236 free high-throughput sequence aligner <sup>51</sup>. The NCBIInr database was used to find the most  
237 probable protein homolog of the closest species to *Gammarus fossarum* for each protein with  
238 an e-value below  $10^{-10}$ . The Swissprot database was used to find the top 5 closest proteins in  
239 terms of sequence identity to define for each identified protein their biological processes and  
240 molecular functions as previously described <sup>52</sup>, selecting a bit score alignment above 20 and  
241 an e-value below  $10^{-4}$ .

242

### 243 *Differential proteomics*

244 The quantities of each protein in animals exposed to treated intracellular or cell wall  
245 compartments vs non-treated intracellular or cell wall compartments respectively were  
246 compared using Patternlab version 4.0 <sup>53</sup>. The L-stringency was set at 0.6. At least four  
247 measurements out of the five replicates per condition were taken into account. The selected  
248 data were normalized with the total signal. Proteins were distinguished into 4 groups: the first  
249 one corresponds to proteins that satisfied both the F-stringency (<0.30) and statistical criterion  
250 (FDR < 0.05); the second one was filtered out by their L-stringency because of the low  
251 protein abundance; the third one groups proteins that meet only the fold criterion, and the  
252 fourth one comprises proteins that did not satisfy both criteria. Proteins from the first group  
253 were selected for the Gene Ontology (GO) slim differential analysis. The average percentage

254 of S-containing residues in proteins significantly modulated, either over-detected or less-  
255 detected, for each comparative test was calculated without specific weight.

256

### 257 *GO slim differential analysis*

258 GO slim analysis was performed taking the sum of SC of each GO slim related proteins for  
259 each sample. Univariate statistical analysis was performed to define, with the Ttest pValue,  
260 the reliability between conditions taking into account the six replicates. The ratio R for each  
261 GO slim was calculated for comparing treated conditions and non-treated conditions, either  
262 for the intracellular or the cell wall compartment, as the sum of SC of all replicates in the  
263 treated samples divided by the sum of SC of all replicates in the control samples. The ratio  
264 was compared using “prcomp” from the basic stats package of R to perform a Principal  
265 Component Analysis (PCA). From the PCA, significant GOSlim contributing for more than  
266 50 % to distinguish each condition (contrib > 0.5) were extracted.

267

## 268 **Results and discussion**

### 269 *Accumulation and subcellular distribution of Hg in G. fossarum*

270 THg concentrations in subcellular fractions were 2 to 6x higher in animals fed with cell wall  
271 compartments than intracellular compartment of MeHg pre-exposed plants, and these  
272 differences were significant ( $p < 0.05$ ; Figure 2). For the high and low MeHg treatment  
273 respectively, THg in MSF was 2.9x and 2.0x higher, while BDM 5.8x and 2.3x higher for  
274 individuals fed with cell wall than intracellular compartments. To gain insights into transfer  
275 dynamics of MeHg in *G. fossarum*, we analyzed by differential centrifugation the subcellular  
276 distribution of THg and MeHg (see below) in 3 fractions (Figure 2). In controls and low  
277 MeHg treatments,  $42.5 \pm 6.5\%$  of THg was found in S2 and an equal concentration in MSF and  
278 BDM fractions ( $30.0 \pm 6.1\%$ ). For gammarids fed with the high MeHg intracellular  
279 compartment, the MSF increased ( $41.0 \pm 5.7\%$ ), while for the high MeHg cell wall treatment  
280 the proportion of THg significantly increased in the BDM ( $50.0 \pm 2.8\%$ ;  $p < 0.05$ ). We analyzed  
281 the spectrograms obtained by HPLC (Figure S1). For example, 5 peaks appeared visually  
282 increased and 4 decreased in high MeHg vs ctl cell wall compartments (Figure S1). Because  
283 individual peaks are poorly defined by HPLC method used here because bias are caused by  
284 the overlapping of peaks, we compared the area of peaks measured at 280 nm in HPLC  
285 eluting fractions normalized by the biomass of animals: the area of MTLPs-MW was  
286 increased significantly ( $p > 0.05$ ) by  $1.7 \pm 0.1$  vs controls in both cell wall treatments and low  
287 intracellular treatments. Despite visual differences in peaks (Figure S1), no significant

288 differences were found for the sum of areas in HMW and LMW between treatments and  
289 control. Sequestration of toxic metal by MTs and granules is a very common strategy in biota  
290 to control intracellular metal speciation and thus toxicity<sup>17, 18</sup>. MeHg in bivalves and several  
291 fish preys was hypothesized to be bound in the intracellular fraction to heat-stable-protein<sup>54</sup>,  
292<sup>55</sup>. THg partition between the sensitive (HeSP; 40%) and detoxified (MTLPs-MW; 60%)  
293 fractions was previously observed in the cytosol of *C. riparius* exposed to IHg spiked  
294 sediments<sup>37</sup>. In the same line, during a 7 day-long depuration phase, both IHg and MeHg  
295 concentrations in *G. fossarum* feeding on IHg or MeHg pre-exposed *E. nuttallii* did not  
296 significantly vary<sup>10</sup>, supporting that in gammarids the accumulated MeHg was bound  
297 strongly to biological ligands, i.e. MTs and granules<sup>56, 57, 58</sup>. Here, this detoxification strategy  
298 could allow gammarids to reduce the toxicity of the MeHg entering their cytosol when the  
299 concentration increased.

300 Ratio of MeHg as THg was 111±17 % in S2 and S4, 54.3±3.7% in P2 and 60.0±3.8% in P3.  
301 Consequently, MeHg proportion as THg increased from 68 to 95% similarly in BDM and  
302 MSF in control to high MeHg treatments, respectively (n=3; Table S2). In our previous study  
303 with similar feeding of gammarids with *E. nuttallii*<sup>10</sup>, bioaccumulation measured on whole  
304 individuals hindered to determine where the Hg demethylation took place, e.g. in plant tissues  
305 before feeding (for instance, because of plant material decay or biotransformation by  
306 microbes), or during digestion or metabolization in gammarids. Here, data supported the  
307 second hypothesis, in line with observations made in *C. riparius* of an intracellular  
308 demethylation in the insoluble fraction<sup>11</sup>. These observations are consistent with a  
309 generalized demethylation of MeHg in experimental food chains formed by algae (70%) and  
310 zooplankton (13%)<sup>59</sup>, but contrast in natural food chains with observed MeHg  
311 biomagnification<sup>2, 31</sup>.

312

### 313 ***Impact of dietary MeHg on proteome in G. fossarum***

#### 314 *Number and pattern of modulated proteins*

315 We analyzed the whole proteome extracted from animals by shotgun proteomics and  
316 interpreted the high-resolution tandem mass spectra using a *G. fossarum* RNA-seq derived  
317 database. This proteogenomic approach led to 375,286 peptide-to-spectrum matches  
318 corresponding to 12,719 unique peptide sequences, resulting in 3,261 proteins identified with  
319 at least 2 peptides (Table S3). Globally, a total of 151 gammarid proteins were significantly  
320 modulated for the 4 treatment groups vs the control (Table S3). Among them, 78 were found  
321 more abundant, 67 were less abundant and 4 showed a higher abundance in intracellular but

322 were less abundant in cell wall treatments. Four distinct profiles of differentially abundant  
323 proteins were identified for each treatment (Figure 3). However, a higher overlap (6-8% of  
324 proteins) was observed in the same compartment, while only few proteins were shared  
325 between intracellular and cell wall fed gammarids (Figure 3A). Despite inter-individual  
326 variability is the main component of proteome variability in the organism analyzed in this  
327 study (Supplementary Figure 1) and as previously discussed <sup>44</sup>, the differential analysis  
328 allowed the identification of rather distinct protein profiles for each treatment (Figure 3B).

329 The treatment affecting the higher number of proteins was the high MeHg intracellular  
330 treatment that modulated the abundance of 71 proteins, in line with the higher % of THg  
331 found in MSF. The low MeHg cell wall and intracellular compartments modulated 45 and 44  
332 proteins respectively. The high MeHg cell wall treatment modulated 36 proteins, suggesting  
333 together with the higher proportion of THg in BDM, the occurrence of a higher detoxification.

#### 334 *Functional analysis of modulated proteins*

335 The highest measured upregulation (Fold change 55x and 52x) and downregulation (-17x, -  
336 28x) were observed for proteins involved in 'cytoskeleton' for both cell wall treatments  
337 (Table 1). The Mass of these proteins are predicted between 42-35 kDa and could be involved  
338 in the visual increase of peaks in HMW on HPCL spectrograms (Figure S1), although because  
339 the area of HPLC eluting fractions is not strictly quantitative this comparison has to be made  
340 with caution. However, these proteins are known targets of both MeHg and IHg toxicity  
341 across taxa. For example, in copepods exposed to 50  $\mu\text{g}\cdot\text{L}^{-1}$  IHg in water, authors proposed  
342 that proteins assigned to this GO term (e.g. myosin) were involved in the cell reorganization  
343 to withstand Hg toxicity <sup>60</sup>. Proteins involved in cytoskeleton (actin, tubulin, keratin) that are  
344 essential for vesicle trafficking were significantly modified in fish and mammals exposed to  
345 IHg and MeHg supporting cytoskeleton disruption due to metal exposures <sup>61, 62, 63, 64, 65</sup>. In  
346 tuna muscle, binding of Hg to Beta-actin encompassed approximately 34% of Hg in the fish  
347 muscles<sup>66</sup>. Authors hypothesized that Hg binds to thiols in proteins. Here we observed that S  
348 represented  $4.27 \pm 0.08$  % of residues in all samples (Table S4, Figure S3), not pointing to an  
349 increase of thiols in proteome of animals fed with Hg treatments.

350 Several modulated GO terms such as development/growth, energy metabolism, reproduction,  
351 locomotion/motility were common in all treatments (Table 2). To compare the impact of  
352 treatments on *G. fossarum* at a higher system level, we identified the most influent GOslim in  
353 the PCA as being autophagy (148), mitochondrion organization (18), ribosome biogenesis  
354 (164) and sulfur compound metabolic processes (1; Figure 4). Indeed, MeHg cell wall and

355 intracellular treatment decreased and increased the level of those GO terms respectively  
356 (Table 3), suggesting a different impact of the cell wall treatment than intracellular treatment  
357 on *G. fossarum* cell metabolism. GOslim identified for gammarids fed with MeHg from the  
358 intracellular compartment supported the induction of the cellular machinery likely for  
359 replacement of damaged proteins and warranting cell functions, while in animals fed with the  
360 cell wall compartment GOslim pointed mitochondrial perturbations and the induction of  
361 autophagy, ligase and nuclease activities supporting a perturbation of cell organization  
362 triggering repair pathways. In the same line, the contrasted modulation of GO terms related to  
363 gene expression (ribosome, RNA, DNA), protein maturation (ligase and transferase activities)  
364 and growth/development supports a higher modulation of proteome by intracellular MeHg,  
365 but a more detrimental impact to the cell metabolism of the cell wall MeHg. As such, the  
366 tolerance mechanisms triggered by the high-MeHg cell wall treatment resulting in a higher  
367 proportion of THg in BDM appeared to have a cost on fitness for those animals. Data are  
368 congruent with studies on one wild *G. fossarum* population exposed historically to Cd ,  
369 evidencing the fitness costs and modification of toxicological sensitivities <sup>67</sup>.

370 Here, differentially detected GO terms are in line with several known effects of MeHg. As  
371 mentioned above, the cytoskeleton participates in the transport of molecules and signaling by  
372 vesicular trafficking involved in autophagy <sup>68, 69</sup>. Autophagy is involved in elimination of  
373 aberrant or obsolete cellular structures. Mitochondria are known target of MeHg in animals <sup>63</sup>,  
374 <sup>70</sup>. For example, in shrimps <sup>203</sup>Hg exposure by diet resulted in Hg bioaccumulation in  
375 organelles (10%) and HeSP (40%), while in *Crassostrea virginica* a 4 week-long exposure to  
376 0.056  $\mu\text{M}$  Hg through water resulted in 16x higher concentration in organelles than MTLPs  
377 <sup>71,72</sup>. Besides, MeHg was shown to depolarize mitochondrial membrane in synaptosomes <sup>73</sup>.  
378 Here, bioaccumulation in mitochondria was not investigated, but this GO term supported an  
379 interaction of Hg with this organelle in the high MeHg cell wall treatment. The GO term  
380 “gene regulation” was also abundant in copepods exposed to 50  $\mu\text{g}\cdot\text{L}^{-1}$  IHg in water and in  
381 Medaka exposed to 1 and 10  $\mu\text{g}\cdot\text{L}^{-1}$  IHg in water <sup>60, 64</sup>.

382 On the other hand, some expected GO terms were not found as significantly different, notably  
383 proteins involved in reduction-oxidation (redox) metabolism and MTs in opposition with  
384 observation of a predominant BDM distribution for the high MeHg cell wall treatment.  
385 MeHg, IHg and other metals induce ROS production. Here, antioxidant enzymes that are  
386 often observed in response to IHg exposure, e.g. superoxide dismutases, peroxiredoxins and  
387 catalases <sup>63, 64</sup>, were not significantly modulated at the protein level. Similarly, a proteomic

388 analysis of rats exposed to IHg by diet did not identify GO terms directly linked to redox,  
389 despite the fact that they measured significantly increased lipid peroxidation by classical  
390 biochemical analyses<sup>63</sup>. Nevertheless, here the GO term sulfur compound metabolic process  
391 related to all chemical reactions and pathways involving sulfur-containing compounds (e.g.  
392 methionine, cysteine, glutathione) strongly influenced the distribution in the PCA among  
393 intracellular samples, while the proportion of Cysteine and Methionine residues in modulated  
394 proteins didn't changed among treatments. This suggests the preexistence in the cytosol of a  
395 sufficient pool of MTLP-MW proteins that could be involved in Hg-chelation in cytosol, as  
396 also supported by the quite stable S proportion in residues of proteins for all treatments (Table  
397 S4, Figure S3). Similarly, *G. fossarum* specimens collected *in situ* from a clean and a  
398 contaminated river in Croatia showed increased soluble metal levels in gammarids in the  
399 contaminated site, but similar MT concentrations in both sites<sup>74</sup>. In the same line,  
400 *Caenorhabditis elegans* chronically exposed to 60 mg·L<sup>-1</sup> MeHg showed no increase in mt  
401 gene expression level after 15h exposure, while *mt*-gene loss-of-function mutants were  
402 markedly more sensitive than wildtype to MeHg<sup>75</sup>. Nevertheless, previous studies in fish  
403 showed that MTs correlated to Hg concentration in liver, but not muscles and gills<sup>76</sup>. Here the  
404 proteomic analysis was conducted on whole organisms, potentially masking putative organ-  
405 specific regulation. More investigation, outside the scope of the current study would be  
406 necessary to conclude on the role of MTs in the tolerance to dietary MeHg in our  
407 experimental conditions.

408 In conclusion, our analysis supported a sublethal stress likely coped by *G. fossarum* according  
409 to the relative low number of modulated proteins in our experimental conditions. The analysis  
410 revealed a correlation between protein abundance and Hg subcellular bioaccumulation in  
411 MSF, but a different protein profile for each concentration, suggesting the occurrence of  
412 several thresholds of regulation of the proteome. Data also suggested that in response to  
413 increasing doses of MeHg, *G. fossarum* activated and/or deactivated some biochemical  
414 processes to compensate the loss or impairment of other pathways caused by the toxicity of  
415 MeHg. As such, defense mechanisms triggered by dietary MeHg appeared to have a cost for  
416 the fitness of animals. Our experiment brought thus new insights on the specific effects of  
417 MeHg in diet at sublethal concentration.

418 Our data in addition confirmed that Hg bound in cell walls of plants can be assimilated by *G.*  
419 *fossarum*, which is consistent with its feeding strategy. The cell wall constituents are expected  
420 to remain for a long period in the environment because of their recalcitrant nature. Here, data  
421 point cell walls as a potential source of Hg transfers and toxicity to primary consumers.

422 Moreover, proteomic analysis revealed contrasted responses for intracellular and cell wall  
423 treatments, congruent with detoxification mechanisms and supporting that the different  
424 binding sites in shoots consequently caused different toxicity to consumers. In this context  
425 feeding behaviour of consumers appears instrumental for the fate and impact of Hg in food  
426 chains. In particular, the abundance of species and their feeding strategy need to be  
427 considered to adequately protect aquatic systems, as both will significantly influence the fate  
428 of Hg in local food webs. For example, the abundance of macrophytes in shallow water  
429 systems might favor the transfer of MeHg to food webs.

430

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436 more integrative approaches.

437

### 438 **Supporting Information.**

439 Table S1: Hg concentrations in water, *E. nuttallii* and *G. fossarum*; Table S2: Hg  
440 concentration in subcellular fractions; Table S3: list of identified proteins; Tables S4 to S6:  
441 proportion of thiols in proteins; Figure S1: HPLC chromatograms, Figure S2: Principal  
442 Component Analysis and Hierarchical clustering of samples, Figure S3: proportion of thiols in  
443 proteins against fold changes.

444

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**Table 1.** List of the five most up and down-detected proteins in each treatment. Abundance vs control (Log2 fold change), annotation, % identities and e-value are listed. Values in bold are significantly different to control (p-value <0.05 calculated with univariate statistic: Ttest).

<b>Functional annotation</b>	<b>% sequence identity</b>	<b>Expect value</b>	<b>I-low</b>	<b>I-high</b>	<b>C-low</b>	<b>C-high</b>
<b>Cell structure</b>						
actin, cytoplasmic 1	51.3	1.5e-58	1.1	-1.8	<b>55.2</b>	<b>53.0</b>
myosin light chain alkali-like	53.8	5.9e-23	-2.3	<b>-5.3</b>	2.2	1.7
tubulin alpha-3 chain	75.2	7.1e-135	5.1	<b>18.1</b>	-1.7	-1.7
myosin heavy chain, muscle-like isoform X18	76.1	1.7e-70	1.1	6.1	<b>-11.5</b>	<b>-4.9</b>
myosin heavy chain type b	58.7	2.6e-69	-1.0	5.9	<b>-7.1</b>	<b>-7.0</b>
collagen alpha-1, IV, chain precursor, putative	63.5	1.2e-61	-1.7	-2.7	-2.3	<b>-7.9</b>
myosin heavy chain, partial	83.8	2.9e-79	2.2	17.1	<b>-17.3</b>	<b>-28.3</b>
<b>Energy metabolism</b>						
ADP/ATP translocase 3-like	94	1.4e-44	<b>-8.0</b>	-2.2	7.2	5.7
fructose-bisphosphate aldolase	85.7	5.8e-31	<b>7.0</b>	<b>2.7</b>	1.1	-1.0
sucrase-isomaltase, intestinal	59.8	9,00E-102	<b>-6.4</b>	-2.6	-2.1	-1.1
glycogen phosphorylase	67.4	2.5e-98	<b>6.4</b>	<b>8.6</b>	-1.9	-1.2
aconitate hydratase, mitochondrial	91.6	2.4e-168	3.9	<b>6.5</b>	1.4	-1.2
endoglucanase E-4	72	5.1e-147	<b>-4.4</b>	<b>-5.4</b>	-3.0	-1.2
malate dehydrogenase, cytoplasmic	87.8	4.1e-161	<b>3.9</b>	<b>6.0</b>	-1.1	-1.6
enolase isoform X1	77.7	1.7e-113	<b>-14.1</b>	-3.0	-6.0	-5.1
family 7 cellobiohydrolase	77.9	6.7e-61	1.4	-8.9	-2.1	<b>-7.9</b>
<b>Response to stress</b>						
endoplasmin-like isoform X1	78.5	5.2e-177	<b>3.4</b>	2.2	<b>6.8</b>	<b>7.1</b>
oplophorus-luciferin 2-monooxygenase non-catalytic subunit	34	4.4e-46	2.1	1.7	<b>5.3</b>	3.3
glucosylceramidase-like	73.6	4.5e-56	<b>-4.1</b>	<b>-6.2</b>	2.1	2.1
phosphate carrier protein, mitochondrial-like	81.3	2.5e-119	-2.0	<b>-7.5</b>	1.2	1.5
hemolymph clottable protein-like isoform X1	31.6	1.1e-06	<b>4.3</b>	3.1	2.8	1.3
putative glucosylceramidase 3	63.8	7.9e-42	-1.3	-1.2	<b>4.5</b>	1.0



dehydrogenase/reductase SDR family member 4	56.1	3.5e-45	<b>4.4</b>	-1.3	1.8	-1.0
beta-1,3-glucan binding protein	38	3.7e-41	-1.5	3.1	<b>-6.5</b>	<b>-8.6</b>
endoplasmin-like isoform X2	70	5.5e-135	-1.0	2.4	<b>-8.8</b>	<b>-8.7</b>
<b>Others</b>						
putative glucosylceramidase 3	55.8	6.2e-115	1.1	-2.5	2.5	<b>6.2</b>
putative ferric-chelate reductase 1 homolog	30.3	1.7	1.3	-3.1	4.3	<b>5.2</b>
uncharacterized protein	49.5	9.2e-43	1.1	4.0	2.3	<b>15.3</b>
uncharacterized protein	29.9	7.9e-55	1.1	-1.2	<b>4.5</b>	2.9
uncharacterized protein	56	4.2e-61	<b>6.7</b>	<b>7.5</b>	3.2	1.7
uncharacterized protein	61.9	1.4e-78	-1.9	<b>-4.7</b>	-1.7	1.1

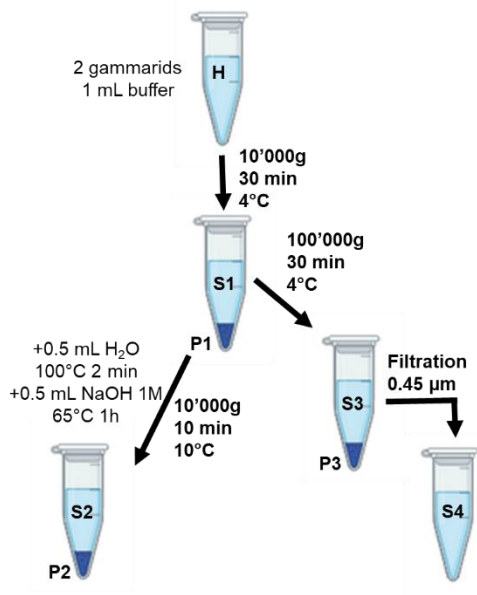
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**Table 2.** Ratio of GOslim terms for molecular function (MF), biological process (BP) and cellular compartment (CC) showing opposite trends for *G. fossarum* feeding on the low or high MeHg contaminated intracellular (I) or cell wall (C) compartment vs respective controls of *E. nuttallii*. Values in bold are significantly different to control (Log2 fold change, \*p-value <0.05 calculated with univariate statistic: Ttest).

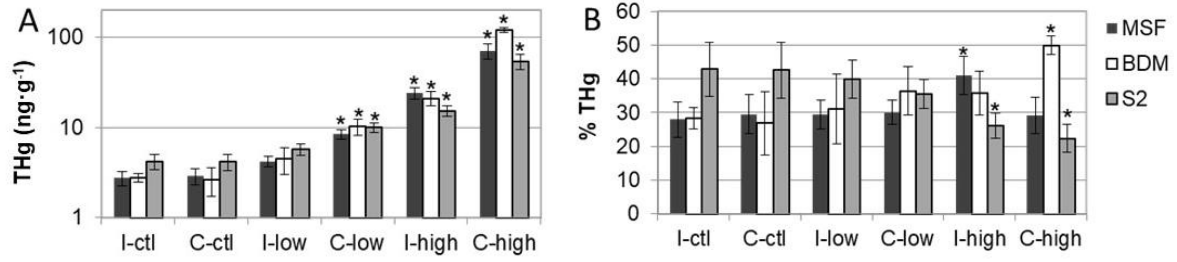
		<b>I-low</b>	<b>I-high</b>	<b>C-low</b>	<b>C-high</b>
GOslim_MF	cytoskeletal protein binding	<b>3.7*</b>	<b>1.7*</b>	-3.0	-1.1
GOslim_MF	enzyme binding	<b>2.0*</b>	1.7	-1.8	-2.0
GOslim_MF	DNA binding	<b>3.0*</b>	1.9	<b>-2.2*</b>	<b>-1.1*</b>
GOslim_MF	ATPase activity	<b>3.0*</b>	1.3	-2.0	-1.2
GOslim_BP	plasma membrane organization	<b>3.0*</b>	<b>1.7*</b>	<b>2.5*</b>	-2.8
GOslim_BP	anatomical structure development	<b>1.1*</b>	1.1	-2.0	<b>-1.1*</b>
GOslim_BP	anatomical structure formation involved in morphogenesis	<b>3.7*</b>	<b>1.6*</b>	-1.4	<b>-1.4*</b>
GOslim_BP	circulatory system process	<b>2.9*</b>	1.5	-3.6	-2.3
GOslim_BP	embryo development	<b>3.6*</b>	1.5	-3.2	<b>-1.8*</b>
GOslim_BP	reproduction	<b>1.7*</b>	1.5	-1.9	<b>-1.5*</b>
GOslim_BP	growth	<b>1.1*</b>	<b>2.0*</b>	-2.8	-2.4
GOslim_BP	locomotion	<b>1.7*</b>	1.4	-1.3	<b>-1.4*</b>
GOslim_BP	cell motility	<b>3.8*</b>	1.4	-2.2	<b>-1.5*</b>
GOslim_CC	chromosome	<b>4.2*</b>	1.1	-3.7	-1.2
GOslim_CC	cytosol	<b>1.0*</b>	<b>1.3*</b>	-1.5	-1.3
GOslim_CC	nucleoplasm	<b>1.4*</b>	1.8	<b>-1.3*</b>	<b>-2.3*</b>
GOslim_CC	microtubule organizing center	<b>5.5*</b>	6.0	2.9	-2.8
GOslim_CC	protein-containing complex	<b>1.1*</b>	1.3	-1.2	<b>-1.1*</b>

**Table 3.** Ratio of GO terms responding to concentration for *G. fossarum* feeding on the low and high MeHg contaminated intracellular (I) and cell wall compartment (C) vs respective controls of *E. nuttallii* (Log2 fold change; \*p-value <0.05; #N/A GO term not found for the treatment).

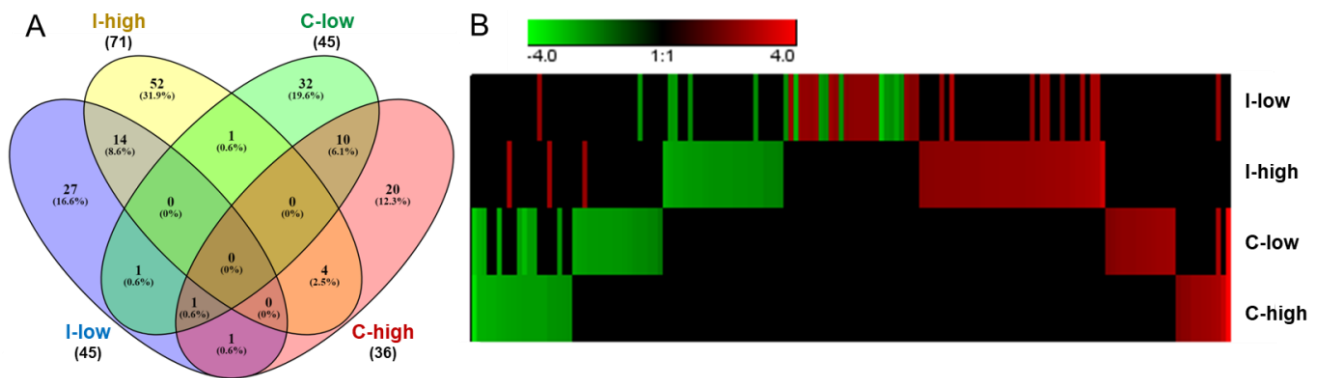
		<b>GO Term # on PCA</b>	<b>I-low</b>	<b>I-high</b>	<b>C-low</b>	<b>C-high</b>
GOslim_BP	autophagy	148	-5.1*	0.8	-0.2	4.8
GOslim_BP	mitochondrion organization	18	-2.9*	0.6	-4.4	4.8
GOslim_BP	ribosome biogenesis	164	#N/A	2.4	-5.6	#N/A
GOslim_CC	microtubule organizing center	198	5.5*	6.0	2.9	-2.8
GOslim_CC	ribosome	207	#N/A	2.4	-5.6	-4.9*
GOslim_MF	transferase activity, transferring glycosyl groups	5	5.9*	5.8*	-5.1	#N/A
GOslim_MF	ligase activity	99	0.3	0.7	-4.4	4.8
GOslim_MF	rRNA binding	101	#N/A	2.1	-4.8	-4.9*
GOslim_MF	structural constituent of ribosome	75	#N/A	2.4	-5.6	-4.9*



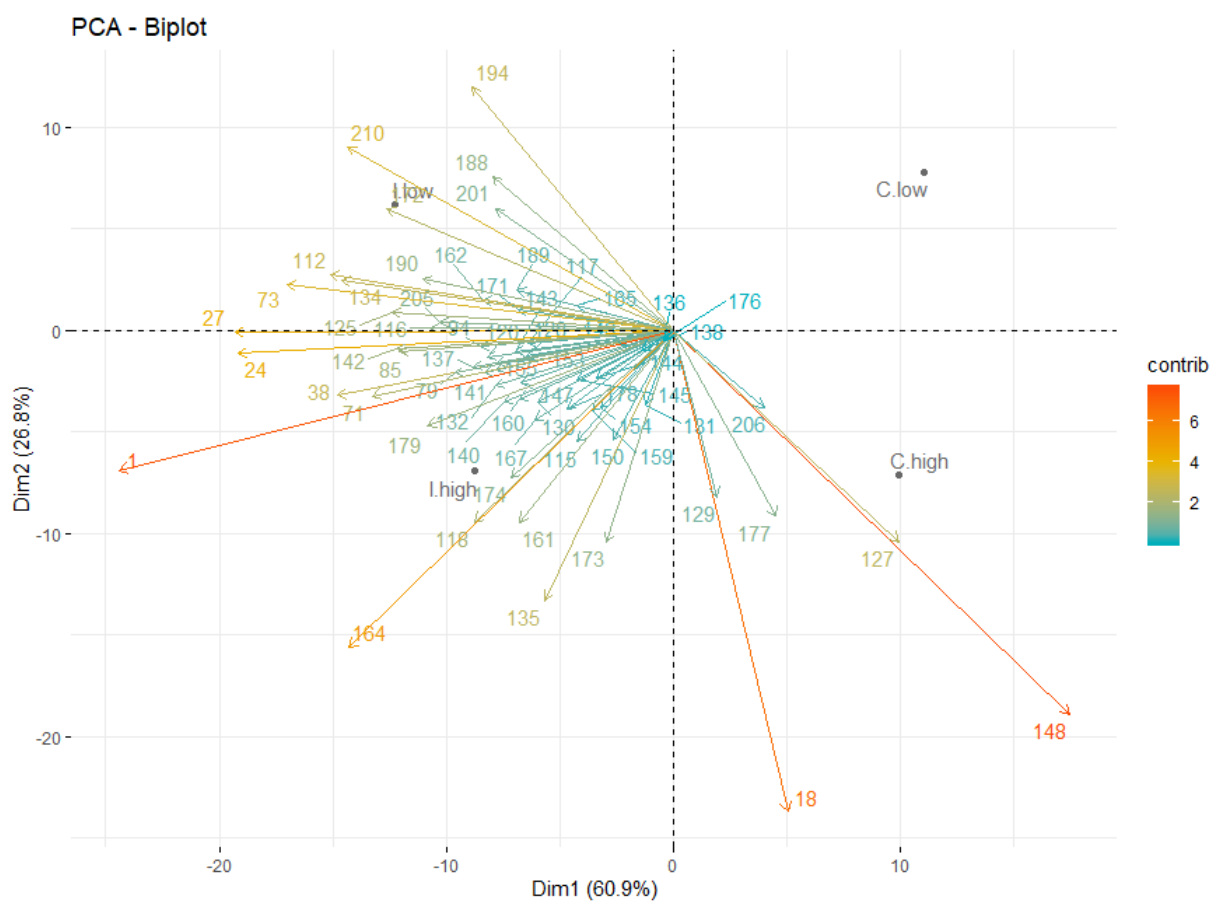
**Figure 1.** main steps of differential centrifugation protocol.



**Figure 2.** Concentration ( $\text{ng}\cdot\text{g}^{-1}$ ; mean  $\pm$  sd; A) and proportion (%; B) of THg measured in MSF, BDM and debris (S2) for *G. fossarum* feeding on the MeHg contaminated intracellular (I) or cell wall (C) compartment of *E. nuttallii* (\* $p < 0.05$  vs control;  $n = 3$ ).



**Figure 3:** Venn diagram (A) and heatmap (Log<sub>2</sub> fold change) (B) of differentially abundant proteins in *G. fassarum* feeding 7 days on the low or high MeHg contaminated intracellular (I) or cell wall (C) vs control (p-value <0.05). Maximum of heatmap is set to 4 for visibility of smaller fold changes (see Table 1 for identification of most modulated proteins).



**Figure 4.** PCA of GOslim terms according to biological processes in *G. fossarum* feeding 7 days on the low or high MeHg contaminated intracellular (I) or cell wall (C) vs control.