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### ► To cite this version:

Clément Baratange, Hugo Baali, Véronique Gaillet, Isabelle Bonnard, Laurence Delahaut, et al.. Bioaccumulation and molecular effects of carbamazepine and methylmercury co-exposure in males of *Dreissena polymorpha*. *Science of the Total Environment*, 2023, 897, pp.165379. 10.1016/j.scitotenv.2023.165379 . hal-04161067

**HAL Id: hal-04161067**

**<https://normandie-univ.hal.science/hal-04161067v1>**

Submitted on 22 Jul 2023

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1 **Bioaccumulation and molecular effects of carbamazepine and methylmercury co-exposure**  
2 **in males of *Dreissena polymorpha***

3

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23 **Abstract**

24 *Dreissena polymorpha* is a bivalve promising for biomonitoring in freshwater ecosystems thanks to its  
25 abundance and high filtration activity allowing rapid uptake of toxicants and identification of their  
26 negative effects. Nonetheless, we still lack knowledge on its molecular responses to stress under  
27 realistic scenario, *e.g.* multi-contamination. Carbamazepine (CBZ) and Hg are ubiquitous pollutants  
28 sharing molecular toxicity pathways, *e.g.* oxidative stress. A previous study in zebra mussels showed  
29 their co-exposure to cause more alterations than single exposures, but molecular toxicity pathways  
30 remained unidentified.

31 *D. polymorpha* was exposed 24h (T24) and 72h (T72) to CBZ ( $6.1 \pm 0.1 \mu\text{g L}^{-1}$ ), MeHg ( $430 \pm 10 \text{ ng L}^{-1}$ )  
32 and the co-exposure ( $6.1 \pm 0.1 \mu\text{g L}^{-1}$  CBZ and  $500 \pm 10 \text{ ng L}^{-1}$  MeHg) at concentrations representative of  
33 polluted areas ( $\sim 10 \times$  EQS). RedOx system at the gene and enzyme level, the proteome and the  
34 metabolome were compared. The co-exposure resulted in 108 differential abundant proteins (DAPs),  
35 as well as 9 and 10 modulated metabolites at T24 and T72, respectively. The co-exposure specifically  
36 modulated DAPs and metabolites involved in neurotransmission, *e.g.* dopaminergic synapse and  
37 GABA. CBZ specifically modulated 46 DAPs involved in calcium signaling pathways and 7 amino acids  
38 at T24. MeHg specifically modulated 55 DAPs proteins involved in the cytoskeleton remodeling and  
39 hypoxia-induced factor 1 pathway, without altering the metabolome. Single and co-exposures  
40 commonly modulated proteins and metabolites involved in energy and amino acid metabolisms,  
41 response to stress and development. Concomitantly, lipid peroxidation and antioxidant activities  
42 were unchanged, supporting that *D. polymorpha* tolerated experimental conditions.

43 The co-exposure was confirmed to cause more alterations than single exposures. This was attributed  
44 to the combined toxicity of CBZ and MeHg. Altogether, this study underlined the necessity to better  
45 characterize molecular toxicity pathways of multi-contamination that are not predictable on  
46 responses to single exposures, to better anticipate adverse effects in biota and improve risk  
47 assessment.

48 **Keywords:** bivalve, freshwater, multi-omics, toxicity pathways

49        **1. Introduction**

50        In the context of environmental contamination, biomonitoring aims to assess environmental  
51 health to protect biota and linked ecosystem services. The zebra mussel *Dreissena polymorpha*  
52 (Pallas, 1771) is promising for freshwater biomonitoring and ecotoxicological studies (Kerambrun et  
53 al., 2016; Klimova et al., 2017; Louis et al., 2019, 2020; Hani et al., 2021; Baratange et al., 2022). This  
54 species is widely distributed, abundant, and sessile. Its high filtration activity results in the  
55 bioaccumulation of pollutants triggering biological responses that can be measured (Binelli et al.,  
56 2015). Nonetheless, a better understanding of the physiology of this mussel is a prerequisite for its  
57 efficient use in biomonitoring.

58        Recent omics techniques opened new research perspectives in stress biology and ecotoxicology,  
59 particularly in non-model biota (Gonzalez and Pierron, 2015; Gouveia et al., 2019). Proteomics and  
60 metabolomics identified molecular targets, molecular toxicity pathways of pollutants, and potential  
61 biomarkers relevant for biomonitoring (Armengaud et al., 2014; López-Pedrouso et al., 2020; Dumas  
62 et al., 2022a). Combining multi-omics approaches and targeted endpoints at different biological  
63 organization levels gives a holistic view of responses and appears particularly powerful to identify  
64 molecular toxicity pathways of pollutants (Brinke, 2017; Gouveia et al., 2019; Dumas et al., 2022a).

65        Here, we investigated molecular toxicity pathways of two ubiquitous pollutants in aquatic  
66 ecosystems: mercury (Hg) and carbamazepine (CBZ) (Andreu et al., 2016). CBZ is an anti-epileptic  
67 drug without European legislation, but Germany and the Netherlands recommended an  
68 environmental quality standard (EQS) of 0.5  $\mu\text{g L}^{-1}$  (EC, 2010; Kase, 2010; ETOX, 2011). Due to its  
69 limited biodegradation and elimination by wastewater treatment plants (Zhang et al., 2008; Calisto et  
70 al., 2011), CBZ is ubiquitous and was proposed as an indicator of the level of anthropogenic activities  
71 (Clara et al., 2004; Dvory et al., 2018). It reached up to 10  $\mu\text{g L}^{-1}$  in freshwaters (Verlicchi et al., 2012).  
72 Concentrations are expected to increase in the future, because of its wide use in human and  
73 veterinary medicine (Oldenkamp et al., 2019). The mode of action of CBZ in vertebrates was

74 suspected to target ion channels (Ambrósio et al., 2002). In bivalves, CBZ exposure triggered  
75 oxidative stress, defense responses and altered the energy metabolism at 1-10  $\mu\text{g L}^{-1}$  range (Chen et  
76 al., 2014; Almeida et al., 2015; Baratange et al., 2022; Dumas et al., 2022b, Baali and Cosio, 2022).  
77 CBZ at 5  $\mu\text{g L}^{-1}$  caused a delayed spermatogenesis in *D. polymorpha* (Magniez et al., 2018), suggesting  
78 reprotoxicity.

79 Hg is a metal ubiquitous in aquatic ecosystems due to natural and anthropogenic sources. It is  
80 included in the European list of priority pollutants (Directive 2008/105/EC) and targeted by the  
81 Minamata International Convention (Reg. EC 1881/2006; <http://mercuryconvention.org/>). The  
82 current EQS in Europe is 70  $\text{ng L}^{-1}$  of total Hg (Directive 2008/105/EC). Recently, up to 1-8  $\mu\text{g L}^{-1}$ ,  
83 representing more than 100x EQS, were reported in aquatic environment (Mahat et al., 2018; Zhou  
84 et al., 2018). Hg occurs mainly as inorganic Hg (IHg; HgII) or methylmercury (MeHg;  $\text{CH}_3\text{HgII}$ ) forms in  
85 the aquatic environment. IHg in 10-30  $\mu\text{g L}^{-1}$  range was reported to cause oxidative stress, alter  
86 energy metabolism and defense responses in bivalves (Liu et al., 2011; Velez et al., 2016; Coppola et  
87 al., 2017). IHg-contaminated algae modulated seven proteins involved in reproductive function of  
88 *Crassostrea angulata*, suggesting reprotoxicity (Zhang et al., 2013). However, MeHg toxicity is of  
89 most concern due to its biomagnification in food webs (Kershaw and Hall, 2019) and lower  
90 depuration than IHg (Metian et al., 2020). In vertebrates, MeHg exposure resulted in alterations of  
91 the nervous system and cytoskeleton (Yadetie et al., 2016; Pierozan et al., 2017). In gills of *D.*  
92 *polymorpha*, exposure to 280  $\text{ng L}^{-1}$  MeHg 7d caused antioxidant responses and cellular damages  
93 (Baratange et al., 2022).

94 CBZ and Hg have been found in common sites in aquatic environments (*e.g.* Zhang et al., 2017).  
95 Both alter similar molecular toxicity pathways in biota. Their co-exposure in *D. polymorpha* caused  
96 more effects than single exposures (Todgham and Stillman, 2013; Baratange et al., 2022). Besides,  
97 effects observed in the co-exposure were unpredictable based on the bioaccumulation and effects  
98 caused by single exposures (Baratange et al., 2022). In this context, a better insight into the

99 molecular toxicity pathways of CBZ and Hg in single and co-exposures is needed for a better  
100 anticipation of adverse effects in complex exposure scenarios. Because gender modulates responses  
101 (Ji et al., 2013, Ji et al., 2014), we exposed males of *D. polymorpha* to CBZ, MeHg and their co-  
102 exposure at nominal concentrations representative of polluted areas (10x EQS; 5  $\mu\text{g L}^{-1}$  and 700  $\text{ng L}^{-1}$ ,  
103 respectively), tolerated by *D. polymorpha* in previous analysis (Baratange et al., 2022). We analyzed  
104 biological responses using untargeted proteomics at 24h (T24) combined with targeted  
105 metabolomics, antioxidant responses at gene and protein levels, lipid peroxidation and pollutant  
106 bioaccumulation at T24 and 72h (T72), to account for kinetics response that might differ among the  
107 different organization levels spanning gene expression to metabolites. We performed a short  
108 exposure to gain knowledge on the mode of action and the molecular toxicity targets of CBZ, MeHg  
109 and the co-exposure in *D. polymorpha*.

110

## 111 **2. Material and methods**

### 112 **2.1. *Dreissena polymorpha* collection and acclimation**

113 About 1,000 individuals of *D. polymorpha* were collected from the “Lac du Der-Chantecoq”  
114 (Marne, France; 48° 36' 22.02" N, 4° 46' 34.0" E) in February 2020. Individuals of 22-28 mm-long  
115 were sorted and cleaned. Mussels were depurated one week in an aquarium filled with 9 L of spring  
116 water (Cristaline Aurèle, table S1) in darkness at 12 °C, corresponding to the field temperature when  
117 collected. Puncture with a syringe in gonads of 0.1 to 0.2mL was observed by microscopy (x200) to  
118 observe gametes, determine sex and select males. The index of sexual maturity (ISM) was  
119 determined on dissected gonad (n = 10) by flow cytometry as described in Magniez et al. (2018): 60%  
120 were in the pre-spawning stage (ISM = 3.5 to 4.7) and 40% in gametogenesis (ISM = 2.5 to 3.5), with  
121 an average ISM of  $3.6 \pm 0.2$ . 360 males were acclimated at 12 °C in darkness for two weeks in 9 L of  
122 local spring water. Males were randomly dispatched into 8 aquaria with 45 individuals per aquarium  
123 and 3.375 L of spring water and further kept in darkness at 12°C for 7d. During this acclimation,

124 mussels were fed with *Nannochloropsis spp.* (Nanno 3600, Instant Algae) using  $2 \cdot 10^6$  of algal cells per  
125 individual per day. Water was continuously aerated ( $> 80\% O_2$ ) and renewed twice a week.

126

## 127 2.2. Experimental design

128 Temperature, feeding and mussel density used during acclimation were maintained for  
129 exposures in semi-static conditions. Water was renewed and spiked daily or not (control, Ctl), with  
130 carbamazepine (CBZ), methylmercury (MeHg) and the co-exposure CBZ+MeHg. Two aquaria were  
131 spiked for each exposure. One was analyzed at 24h (T24), and the other at 72h (T72). No mortality  
132 was recorded during the experiment.

133 The effective concentrations of CBZ and MeHg were determined by sampling and filtering (0.45  
134  $\mu\text{m}$ , Sterivex) 10 mL of water collected daily immediately after spiking ( $n=1$  per tank and per  
135 timepoint). Temperature, pH, conductivity and oxygen concentration, nitrate, nitrite, and ammonium  
136 were respectively analyzed daily with a multimeter (MU 6100H, VWR) and by spectrophotometry  
137 following the manufacturer protocol (Permachem) in a sample of 50 mL water taken just before the  
138 water renewal ( $n=1$ ).

139 At T24, five mussels were deshelled, snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for  
140 proteogenomics analyses. The other endpoints were analyzed at T24 and T72. For bioaccumulation  
141 analyses, 9 mussels were deshelled, rinsed with spring water and the whole soft tissues kept at  $-80$   
142  $^\circ\text{C}$ . For metabolomics analyses, 8 mussels were frozen in liquid nitrogen and stored at  $-20^\circ\text{C}$ . For  
143 qPCR and biochemical analyses, 16 mussels were dissected, and digestive glands, gills and gonads  
144 frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ .

145

## 146 2.3. Pollutant analysis

### 147 2.3.1. Carbamazepine



148 Water aliquots were frozen until analysis. Samples were 10x enriched through centrifugal  
149 vacuum evaporation at 37 °C using a Genevac HT Series (SP Scientific). This preparation step was  
150 validated using CBZ standards and control solutions that were systematically concentrated in parallel  
151 to samples. A High-Performance Liquid Chromatography (HPLC) system (Agilent technologies, 1260  
152 Infinity), consisting of a quaternary pump and a photodiode array detector, allowed the measure of  
153 CBZ concentrations from water samples. To elute the analytes (20 µL), a mobile phase containing  
154 acetonitrile (A) and ultra-pure water (ALPHA Q 18.2 MΩ cm<sup>-1</sup>) with orthophosphoric acid (0.1%) (B)  
155 was used on a reverse-phase Agilent Pursuit XRs 5 C<sub>18</sub> column (5 µm x 250 x 3 mm) at 20 °C. CBZ was  
156 measured at 214 nm after the elution at a flow rate of 0.80 mL min<sup>-1</sup> in gradient condition from  
157 35/65% to 100/0% (v/v) A/B. Analyses were performed in triplicate.

158 Soft tissues were freeze-dried (48h at 0.3 mbar; Vacuubrand GMBH) and grinded for 8min at  
159 30 Hz (MM400, Retsch) with one 8 mm stainless steel bead. After weighing, the dry tissue was  
160 resuspended in 10 mL acetonitrile, 200 µL n-heptane and 2.5 mL ammonium acetate (0.4 M). The  
161 supernatant was recovered by centrifugation (1,620 g, 10min), concentrated under nitrogen flux  
162 evaporation at 40 °C, resuspended in the eluent solution, and 10 µL of the samples injected into the  
163 UPLC-MS/MS (Acquity UPLC Xevo TQ-MS system, Waters). The concentration was determined for  
164 each sample by using a standard curve from 0.5 to 12 ng mL<sup>-1</sup> CBZ, and by adding an internal  
165 standard of 50 ng mL<sup>-1</sup> CBZ-d<sub>10</sub> in the samples. The quality of the analyses was validated by analyzing  
166 blanks.

167

### 168 2.3.2. Methylmercury

169 Water aliquots were acidified with 0.5% v/v HCl (Suprapur, Merck), and kept at 4 °C until  
170 analyzed. Whole soft tissues were freeze-dried, weighed and ground for 8min at 30 Hz (MM400,  
171 Retsch) by an 8 mm stainless steel bead and digested in 30% v/v HNO<sub>3</sub> (Suprapur, Merck) for 12h at  
172 60 °C. MeHg concentration was analyzed by MERX-M (Brooks Rand Instruments) following MeHg

173 procedure (US EPA 1630, 2001). The analytical quality was validated by analyzing blanks and certified  
174 reference materials (DORM-4, TORT-2; NRC – CNRC).

175

## 176 2.4. Shotgun proteomics

### 177 2.4.1. Data acquisition

178 Whole soft tissues were freeze-dried and grinded by bead-beating with two 2 mm-diameter steel  
179 beads in a Precellys instrument (Bertin) operated for 3 times 20s at 5,000 rpm. The powder was  
180 pelleted by centrifugation for 1min at 1,500 g, weighted, and dissolved in 12.5  $\mu$ L of LDS1X (Thermo)  
181 per mg of powder. The mixture was subjected to bead-beating for 6 cycles of 20s at 7,200 rpm,  
182 followed by 30s of ultrasonication with a Hielscher UP50H sonicator operated at 90% amplitude, and  
183 then 4 cycles of bead-beating at 6,500 rpm. Each sample was centrifuged for 3min at 10,000 g to  
184 eliminate debris and the resulting supernatant was diluted by 7 volumes of LDS1X. After  
185 denaturation of the proteins for 5min at 99 °C, 30  $\mu$ L were subjected to denaturing electrophoresis  
186 on a NuPAGE 4-12% gradient gel ran in MES buffer (Thermo) for 5min as recommended (Hartmann et  
187 al., 2014). Each proteome was processed as a single polyacrylamide gel slice and proteolyzed with  
188 trypsin (Promega) in presence of 0.01% ProteaseMAX surfactant (Promega) as described by  
189 Hartmann et al. (2014). The resulting peptides (2  $\mu$ L out of 50  $\mu$ L) were analyzed with a Q-Exactive HF  
190 mass spectrometer (Thermo) operated in data-dependent mode using parameters previously  
191 described (Klein et al., 2016). Peptides were resolved on an Acclaim PepMap100 C18 (3  $\mu$ m, 100 Å,  
192 75  $\mu$ m id x 50 cm) at a flow rate of 0.2  $\mu$ L per min along a 90 min gradient of acetonitrile in presence  
193 of 0.1% formic acid (3.2%-20% in 75min and then 20%-32% in 15min). A dataset of 1,472,640 high-  
194 resolution MS/MS spectra was recorded. The dataset was queried with the MASCOT software  
195 (MatrixScience) in follow-up mode against two complementary databases: a RNA-seq derived  
196 database named Dreissena\_HJ1\_pep\_20170801.fasta, and then the  
197 Dreissena\_polymorpha\_Cosio300seq\_2020-11-11.fasta listing 300 additional protein sequences. A

198 protein was considered as significantly modulated if  $|TFOLD| \geq 1.40$  and  $p\text{-value} < 0.05$ . A total of 162  
199 differential abundant proteins (DAPs) were detected when comparing pollutant exposures to the Ctl.  
200 The mass spectrometry and proteomics datasets acquired on samples are available through the  
201 ProteomeXchange Consortium via the PRIDE partner repository, with the dataset identifier  
202 PXD040345 and 10.6019/PXD040345 [The reviewers may access this currently private dataset using  
203 <https://www.ebi.ac.uk/pride/> website with the username as reviewer\_pxd040345@ebi.ac.uk and  
204 password as aRt4WSai ] (tables S2 and S3).

205

#### 206 2.4.2. Protein annotation and enrichment analyses

207 The dataset showed 2,722 proteins with at least seven spectral counts that were annotated by  
208 homology with the EggNOG v5.0 software (<http://eggnog5.embl.de>) using default parameters (auto  
209 adjust per query, transfer annotation from any ortholog, E-value threshold set at  $10^{-3}$ , minimum of  
210 40% identity and of 20% of query and subject coverage). Identified proteins were analyzed with  
211 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database and gene ontologies (GO)  
212 terms using WEB-based GENE SeT Analysis Toolkit (WebGestalt; <http://www.webgestalt.org/>) for  
213 enrichment analysis (table S4), as it offers the possibility to use our dataset of 2,722 proteins as  
214 background list. To identify the most relevant KEGG pathways or GO terms, redundancy reduction  
215 was performed applying a weighted set cover ( $p\text{-value} < 0.05$ , and  $\geq 2$  DAPs). Eventually, non-relevant  
216 pathways for *D. polymorpha* were removed from the output (e.g. fluid shear stress and  
217 atherosclerosis, ko05418). For each protein, the ratio of normalized spectral abundance factor  
218 (%NSAF) of KEGG pathways and GO terms was calculated as the sum of spectral counts in the five  
219 replicates normalized by its molecular mass in kDa divided by the sum of all NSAF values measured in  
220 the exposure condition as previously described (Christie-Oleza et al., 2012).

221

#### 222 2.5. Targeted metabolomics

223 Freeze-dried soft tissues of animals were collected from shells and weighed. Then, metabolites  
224 were extracted by adding a volume of 900  $\mu\text{L}$  of a solution of ice-cold methanol / chloroform (2/1,  
225 v/v), and the samples were homogenized with a bead-beater (Retsch TM MM301) with two tungsten  
226 beads (3 mm diameter) in a 2 mL microtube. The extracts were kept at  $-20\text{ }^{\circ}\text{C}$  overnight, grinded  
227 again at 25 Hz for 90s. A volume of 600  $\mu\text{L}$  of ultrapure water ( $18.2\text{ M}\Omega\text{ cm}^{-1}$ ) was added in each  
228 extract. After homogenization, samples were centrifuged (4,000 g, 10min,  $4\text{ }^{\circ}\text{C}$ ), and the supernatant,  
229 containing metabolites, was collected and kept at  $-20\text{ }^{\circ}\text{C}$  until analyses.

230 Three different aliquots of 180, 120 or 90  $\mu\text{L}$  were pipetted from samples having dry masses of  
231 50 – 90 mg, 90 – 150 mg, and  $> 150$  mg, respectively to reliably detect and quantify metabolites.  
232 Extracts were transferred into 300  $\mu\text{L}$  glass vials and dried for 1h at  $32\text{ }^{\circ}\text{C}$  (Genevac, miVac DNA). Dry  
233 residues were resuspended in 30  $\mu\text{L}$  of methoxyamine hydrochloride (Sigma-Aldrich,  $25\text{ mg L}^{-1}$ ) in  
234 pyridine (Fisher chemical), and automatically homogenized at  $40\text{ }^{\circ}\text{C}$  for 60min, before adding 30  $\mu\text{L}$  of  
235 N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA, Sigma-Aldrich) followed by incubation at  $40\text{ }^{\circ}\text{C}$  for  
236 60min. A volume of 1  $\mu\text{L}$  of the supernatant was injected in the Agilent 7890/5977B GC/MS platform.  
237 We used the equipment and settings described by Thiébaud et al. (2021). Chromatograms were  
238 established and analyzed using the software provided by the manufacturer (MS Quantitative  
239 Analysis, Quant-My-Way, Agilent B09.00). Standard samples included 62 reference compounds at 1-  
240 2-5-10-20-50-100-200-500-750-1,000-1,500  $\mu\text{M}$ . Quadratic calibration allowed to accurately  
241 annotate and quantify 31 metabolites in samples (tables S5, S6, S7).

242

## 243 2.6. Gene expression level

244 The relative gene expression level of catalase (cat), glutathione-s-transferase (gst), superoxide  
245 dismutase (sod) and metallothionein (mt) was analyzed in digestive glands, gills and gonads using  
246 actine (act) and ribosomal protein S3 (ps3) as reference genes (table S8). Digestive glands are  
247 important detoxifying organs for chemical exposure, gills are directly exposed to the media, and

248 gonads are essential to ensure the species reproduction, *i.e.* for the survival. RNA was extracted with  
249 TRI Reagent (Euromedex) following the manufacturer protocol. Quality and quantity of RNA were  
250 determined by electrophoresis on 1% agarose gel in Tris Borate EDTA (TBE; 0.5%) buffer and with  
251 Nanodrop (Jenway). Reverse transcription (RT) was performed using 400 ng RNA with the verso cDNA  
252 synthesis kit (Thermo Scientific) and oligodT primers, with a PCR Mastercycle (Eppendorf) at 42°C for  
253 1h followed by 3min at 95°C. Quantitative polymerization chain reactions (qPCR) were conducted  
254 with 3 µL of 1/10 diluted cDNA and Absolute Blue qPCR SYBR Green (Thermo Scientific), using a  
255 CFX96 automaton (BioRad; 95°C 15min, 40 cycles 95°C 10s, 60°C 45s) following the  $2^{-\Delta\Delta Ct}$  method  
256 (Schmittgen and Livak, 2008).

257

## 258 2.7. Biochemical analyses

259 To distinguish targets of sections 2.6. and 2.7., thereafter gene expression levels are mentioned  
260 in lowercase, while enzyme activities are written in uppercase. We determined the protein  
261 concentration, GST activity, lipid hydroperoxides (LOOH) resulting from lipid peroxidation, CAT  
262 activity, SOD activity and MT abundance in digestive glands, gills and gonads as described in  
263 Baratange et al. (2022). Briefly, tissues were weighed and suspended (8/1 v/w) in phosphate buffer  
264 pH 7.4 (50mM, VWR), phenylmethylsulfonyl fluoride (PMSF 1mM, Sigma-Aldrich) and L-serine borate  
265 (1mM, Sigma-Aldrich) as protease inhibitors, grinded at 30 Hz for 90s three times by an 8 mm  
266 stainless steel bead and centrifuged 15min at 3,000g (4 °C). The supernatant (= homogenate) was  
267 collected and kept at -80 °C until analysis.

268 The automated spectrophotometer Gallery (Thermo Scientific) was used to determine the  
269 protein concentration, GST activity and LOOH. Protein concentration was measured at 600 nm after a  
270 5min reaction by the colorimetric method of red Pyrogallol and 1/6 (v/v) of the homogenate, using  
271 calibration curve from bovine serum sCal ( $66.7 \mu\text{g L}^{-1}$ ) diluted 50 to 200x and protein reagents U/CSF  
272 from the manufacturer (Thermo Scientific). GST activity was measured at 340 nm for 4min through

273 the conjugation of 0.9mM CDNB with 1mM reduced glutathione in 0.1M phosphate buffer pH 6.5,  
274 and 1/40 (v/v) of the homogenate. LOOH concentration was determined at 620 nm after a 30min  
275 reaction on 1/46 (v/v) of the homogenate, 139mM Fe II D-gluconate dehydrate and 240mM orange  
276 xylenol, in acidic solution of H<sub>2</sub>SO<sub>4</sub> 40mM, glycerol 1.37M, formic acid 20mM and NaCl 0.9%, with  
277 tert-butyl hydroperoxide (TBH) at 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16μM for calibration.

278 CAT activity was measured by a spectrophotometer (Cary 50, Agilent, Australia) through the  
279 degradation of 14 mM H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich) at 240 nm for 120s on 1/100 (v/v) of the homogenate for  
280 digestive glands, and 1/50 (v/v) of the homogenate for gills and gonads in 50mM phosphate buffer  
281 pH 7.4, using a calibration curve from purified bovine serum CAT at 1.25, 2.5, 5, 10, 15 and 20 U mL<sup>-1</sup>.

282 SOD activity was measured by a spectrophotometer (Spark 10M, TECAN, Austria) after 20min  
283 reaction in darkness, at 340 nm for 30min on 1/85 (v/v) of the homogenate for digestive glands, and  
284 1/43 (v/v) of the homogenate for gills and gonads, with 2.35mM EDTA dihydrate, 1.17mM MnCl<sub>2</sub>,  
285 1mM β-mercaptoethanol, 0.28mM β-NADH in 50mM phosphate buffer pH 7.4. The inhibition of β-  
286 NADH oxidation was followed, and activity was determined in the linear range, using a calibration  
287 curve from purified bovine serum SOD at 0.125, 0.25, 0.35, 0.5 and 0.7 U mL<sup>-1</sup>.

288 MT concentration was measured with a polarograph (797 VA Computrace Metrohm) by  
289 sulfhydryl groups (-SH) determination, as described by Thompson and Cosson (1984). The  
290 homogenate was heat-shocked 15min at 75°C, kept 10min at 4 °C, centrifuged 10min at 15,000 g (4  
291 °C), and the supernatant collected. Analyses were performed at 4 °C in a thermostated cell  
292 containing 10 mL Bridcka reagent, 150 μL of Triton solution, and 30 μL (digestive glands) or 60 μL  
293 (gonads) of the supernatant. Concentrations were determined using a calibration curve of 1, 2, 3, 4,  
294 5, 6 μg standard MT from liver's rabbit (Sigma).

295

296 2.8. Data interpretation and statistical analysis

297 Data are presented as arithmetic mean  $\pm$  standard error of the mean (SEM). A bioaccumulation  
298 factor (BAF;  $L\ kg^{-1}$ ) of MeHg and CBZ was calculated by dividing the concentration in mussels ( $\mu g\ kg^{-1}$ )  
299 by the effective concentration in water ( $\mu g\ L^{-1}$ ). We applied two-way ANOVA analyses to test if  
300 exposure and/or time significantly altered bioaccumulation, gene expression level, biochemical  
301 responses and metabolome. We applied one-way ANOVA to test if exposure significantly altered  
302 proteome in Rstudio software (v 4.0.3.;  $\alpha = 5\%$ ; table S9). Normality and homoscedasticity of  
303 residuals was checked by Kolmogorov-Smirnov's and Bartlett's tests, respectively. We considered a  
304 biological effect if  $p\text{-value} < 0.05$ , and the fold change (FC)  $\geq 1.20$  and  $\leq 0.83$  for gene expression level  
305 and biochemical analyses and  $FC \geq 1.40$  and  $\leq 0.71$  for proteogenomic and metabolomic.

306 Venn diagram was performed with InteractiVenn (Heberle et al., 2015), while principal  
307 component analysis (PCA) and partial least square discriminant analyses (PLS-DAs) were performed  
308 using Metaboanalyst ([www.metaboanalyst.ca](http://www.metaboanalyst.ca)). Heatmaps were constructed using Metaboanalyst to  
309 cluster individuals with average method and measure of Euclidean distance. Pathway analyses on  
310 modulated metabolites were performed with Metaboanalyst using KEGG database in October 2019  
311 ( $p\text{-value} < 0.05$ , and  $\geq 2$  metabolites).

312

### 313 **3. Results**

#### 314 3.1. Water analyses

315 Effective concentrations of carbamazepine (CBZ;  $6.1 \pm 0.1\ \mu g\ L^{-1}$ ), methylmercury (MeHg;  $430 \pm 10$   
316  $ng\ L^{-1}$ ) and the co-exposure ( $6.1 \pm 0.1\ \mu g\ L^{-1}$  CBZ;  $500 \pm 10\ ng\ L^{-1}$  MeHg) and control (Ctl) were stable  
317 from the beginning to the end of the exposure ( $n=3$ ) and represented 12x the recommended EQS for  
318 CBZ (ETOX 2011) and 7x EQS for Hg in Europe (Directive 2008/105/EC; figure S1). Water analysis  
319 showed stable chemical parameters with similar values for all exposures and time points:  
320 temperature  $12.1 \pm 0.3\ ^\circ C$ , pH  $8.5 \pm 0.1$ , conductivity  $500 \pm 20\ \mu S\ cm^{-1}$ , oxygen saturation  $75.8 \pm 0.1\ \%$

321 O<sub>2</sub>, nitrates 9 ± 1 mg L<sup>-1</sup>, nitrites 0.10 ± 0.02 mg L<sup>-1</sup> and ammonium 0.04 ± 0.02 mg L<sup>-1</sup> (figure S1).  
322 Consequently, responses described thereafter are attributed to the effect of pollutants.

323

### 324 3.2. Bioaccumulation

325 CBZ and MeHg in single and co-exposure at 24h (T24) and 72h (T72), showed a significant  
326 bioaccumulation (respectively:  $p < 2E^{-16}$  and  $p = 1.7E^{-6}$ ) and a similar BAF across time (respectively:  
327  $p = 0.511$  and  $p = 0.143$ ) (figure 1). No difference was observed between single and co-exposure for CBZ  
328 ( $p > 0.1$ ) and MeHg ( $p > 0.1$ ), suggesting that differences show inter-individual variability. MeHg  
329 resulted in BAF 44x to 96x higher than CBZ. The other pollutant was not bioaccumulated in single  
330 exposures ( $p > 0.1$ ).

331

### 332 3.3. Proteogenomics

333 The whole proteome of mussels at T24 by shotgun proteomics and the MS/MS spectra was  
334 interpreted using a RNA-seq derived database. Sequences of 4,892 proteins identified with at least  
335 one peptide were grouped into the database MS20-032\_ProtSpec\_2020-11-27 which comprises a  
336 total of 2,224,933 amino acid residues. This database was queried, resulting in the final assignment  
337 of 838,587 MS/MS spectra (FDR 1%). In total, 32,918 unique peptide sequences at high confidence  
338 (FDR below 1%) were identified. These peptides showed the presence of 4,375 proteins with at least  
339 one specific peptide, and a total of 3,183 with at least two peptides (table S2). Their functional  
340 annotation was obtained by sequence similarity search (table S3).

341 An average of 21,410 spectral counts was measured per sample. A cut-off of >6 spectral count  
342 abundances was used to select 2,722 proteins in the dataset, including 2,477 proteins (91%) with at  
343 least one GO-term or one KEGG pathway. Compared to Ctl, CBZ, MeHg and the co-exposure  
344 respectively showed 46, 55 and 108 differential abundant proteins (DAPs) by univariate analyses,



345 suggesting that the co-exposure caused more alterations than single exposures. CBZ (85%), MeHg  
346 (95%) and their co-exposure (97%) showed mainly DAPs more abundant than in Ctl. Only 7 DAPs  
347 were common in all exposures (figure 2), indicating an increase of the biosynthesis of proteins  
348 specific to each experimental condition. In the same line, PCA analyses of the 162 DAPs in at least  
349 one exposure discriminated exposures along the first component explaining 33.5% of the variability,  
350 and pollutants were differentiated along the second component explaining 12.4% of the variability  
351 (figure 2). The highest increase and decrease in abundance of the 162 DAPs vs Ctl ( $\log_2$  fold change  
352 FC) were  $<2$ , indicating limited protein modulations by pollutants (table 1). Annotations of DAPs in all  
353 exposures included proteins involved in response to stress, signal transduction, transcription and  
354 translation processes. Other DAPs modulated to CBZ and MeHg single exposures were involved in  
355 cytoskeleton (*e.g.* spectrin) while MeHg single exposure and the co-exposure modulated DAPs  
356 involved in intracellular transport. This analysis also identified uncharacterized proteins (7.4%),  
357 underlining the need to gain knowledge on the biology and stress responses of *D. polymorpha*.

358       Enrichment analyses of KEGG pathways and GO-terms confirmed that the co-exposure  
359 CBZ+MeHg caused more alterations than single exposures in *D. polymorpha*, shown by a higher  
360 %NSAF and a higher number of DAPs per enriched pathway (figure 2). The co-exposure modulated  
361 DAPs involved in transcription processes (*e.g.* eukaryotic translation initiation factor 3 complex),  
362 intracellular transport, and signaling pathway such as MAPK (mitrogen activated protein kinases)  
363 cascade. CBZ exposure modulated DAPs involved in protein turnover (*e.g.* regulation of protein  
364 ubiquitination) and signal transduction, including the calcium signaling pathway. MeHg exposure  
365 modulated DAPs involved in cytoskeleton organization (*e.g.* barbed-end actin filament capping) and  
366 signaling pathway, including the hypoxia-inducible factor 1 (HIF-1) and Toll and Imd signaling  
367 pathways. All exposures modulated DAPs involved in amino acid metabolism, energy metabolism,  
368 cellular development, signal transduction and response to stress (figure 2). However, most enriched  
369 GO-terms and KEGG pathways were specific to each exposure, in congruence with the Venn diagram  
370 and PCA analysis (figure 2).

371

#### 372 3.4. Targeted metabolomics

373 At T24, univariate analyses compared to Ctl showed that CBZ exposure resulted in significant  
374 depletion of the amount of 5 metabolites, while the co-exposure resulted in a significant ~~increase~~  
375 decrease of the concentration of one metabolite ( $p < 0.05$ , figure 3, tables S4 and S5). Increase or  
376 decrease in metabolite amounts in exposures compared to Ctl ( $\log_2$  FC) were  $\leq 2.1$ , indicating limited  
377 metabolite modulations in our experimental conditions (figure 3). At T72, no modulation was  
378 measured.

379 A high individual variability (figure 3) could mask modulations of metabolites, thus limiting the  
380 power of univariate approaches. Consequently, multivariate analyses were favored and will be  
381 further discussed. PCA analyses did not discriminate metabolic profiles of exposures, hence PLS-DAs  
382 were performed (figure 4). The metabolic profiles in both CBZ and the co-exposure were different  
383 from Ctl at T24, and the latter at T72. These results further supported that the co-exposure triggered  
384 more alterations than single exposures. At T24, CBZ and Ctl were mostly discriminated by component  
385 1, while the co-exposure and Ctl were discriminated by component 2. Metabolites with a VIP  
386 score  $> 1.0$  (*i.e.* most discriminant metabolites) and  $FC > 1.4$  suggested that CBZ and the co-exposure  
387 resulted in the alteration of the concentration of 7 and 9 metabolites, respectively. At T72, the co-  
388 exposure and Ctl were discriminated along components 1 and 2, with 10 metabolites modulated  
389 compared to Ctl. At both timepoints, modulated metabolites were involved in aminoacyl-tRNA  
390 biosynthesis ( $p < 0.01$ ), valine, leucine and isoleucine biosynthesis and degradation ( $p < 0.05$ ). CBZ  
391 exposure at T24 caused the specific alteration of glycine, serine and threonine metabolism ( $p < 0.01$ )  
392 and biotin metabolism ( $p < 0.05$ ). The co-exposure at both timepoints caused the alteration of  
393 pantothenate and CoA biosynthesis ( $p < 0.01$ ). At T24, the co-exposure altered alanine, arginine,  
394 aspartate, glutamate, proline metabolism ( $p < 0.05$ ), arginine biosynthesis ( $p < 0.01$ ), and at T72  
395 glycerolipid metabolism ( $p < 0.01$ ).

396 Omics and targeted analyses revealed molecular toxicity pathways triggered by single and co-  
397 exposures (figure 5). Single exposures and co-exposure at T24 and the co-exposure at T72 caused the  
398 modulation of amino acid metabolism, energy metabolism, and protein turnover. At T24, single and  
399 co-exposures altered the cellular development and response to stress. Globally, data confirmed that  
400 the co-exposure caused more alterations than single exposures.

401

### 402 3.5. Lipid peroxidation and defense responses

403 We analyzed defense responses at the gene and enzyme level and lipid peroxidation in digestive  
404 glands, gills and gonads. No significant modulation vs Ctl was evidenced ( $p>0.05$ ; table 2), supporting  
405 that MT and defense background level in *D. polymorpha* were efficient to tolerate Hg in our  
406 experimental conditions.

407

### 408 3.6. Link of biological endpoints and pollutant bioaccumulation

409 In our experimental conditions, no obvious correlation of bioaccumulation with biological effects  
410 was observed in *D. polymorpha*: the co-exposure did not significantly change bioaccumulation  
411 ( $p>0.1$ ), while proteomics and metabolomics showed a higher number of significant modulations vs  
412 Ctl.

413

414

## 415 4. Discussion

### 416 4.1. Specific molecular toxicity pathways

417 Carbamazepine (CBZ) exposure to concentration representative of contaminated areas caused a  
418 specific enrichment of proteins involved in the KEGG pathway “calcium signaling”. Similarly, in  
419 digestive glands of *Mytilus galloprovincialis*, exposure to  $1 \mu\text{g L}^{-1}$  CBZ 28d resulted in a significant

420 modulation of the expression of genes involved in calcium ion binding, sodium ion transport, and  
421 potassium channels (Mezzelani et al., 2021). In the same line, injection of 1 mg CBZ kg<sup>-1</sup> in the fish  
422 *Solea senegalensis* caused an increase of up to 3x of Na<sup>+</sup>, K<sup>+</sup>-ATPase activity and 1.1x osmolality at  
423 48h, supporting an alteration of osmoregulation (Seifter and Chang, 2017; González-Mira et al., 2016,  
424 2018). Because ion balance is instrumental to maintain osmotic homeostasis, their modulation may  
425 result in alterations of osmoregulation and cellular homeostasis (Seifter and Chang, 2017). Here, CBZ  
426 exposure resulted in the depletion of six amino acids at 24h (T24). As free amino acids are important  
427 osmoregulators in mollusks, this decrease may alter osmotic homeostasis (Viant et al., 2003). In  
428 addition, KEGG pathway “choline metabolism” was enriched. Choline is a precursor of betaine,  
429 another osmoregulator (Ueland, 2011). As such, Ca<sup>2+</sup> channels might be an early toxicity target of CBZ  
430 in *D. polymorpha*, supporting a similar mode of action of CBZ than in vertebrates (Ambrósio et al.,  
431 2002; von Borstel Smith et al., 2007; Fabbri and Franzellitti, 2016).

432 Besides, Ca<sup>2+</sup> is an important secondary messenger (Liu et al., 2007; Puri, 2020), and CBZ  
433 exposure at T24 caused the depletion of seven proteinogenic amino acids and the enrichment of  
434 differential abundant proteins (DAPs) involved in protein turnover, e.g. “negative regulation of  
435 protein ubiquitination”. Similarly, in digestive glands of males of *M. galloprovincialis*, exposure to  
436 0.08 and 8 µg L<sup>-1</sup> 3d caused a higher abundance of proteins involved in RNA transcription and protein  
437 translation (Dumas et al., 2022b). As such, CBZ appeared here to alter signal transduction, and  
438 modulate protein abundance, most likely to cope with the pollutant.

439 Methylmercury (MeHg) exposure to a concentration representative of contaminated areas  
440 resulted in the enrichment of the “epithelium development”, “positive regulation of nematode larval  
441 development” and “barbed-end actin filament capping”, suggesting an alteration of cytoskeleton and  
442 cellular development. MeHg was previously shown to cause an increase of DAPs involved in  
443 cytoskeleton up to 55x in *Gammarus fossarum* exposed by diet to 20 µg L<sup>-1</sup> MeHg 7d (Cosio et al.,  
444 2021). As such, the cytoskeleton appeared as a toxicity target of MeHg in *D. polymorpha*, supporting

445 a similar mode of action than the one reported in vertebrates (Secor et al., 2011; Yadetie et al.,  
446 2016). Although 55 DAPs were observed, no significant modulation appeared at the metabolome  
447 level, suggesting that proteome modulations were sufficient to deal with MeHg in our experimental  
448 conditions.

449 The co-exposure caused the specific enrichment of “dopaminergic synapse” and the depletion of  
450 GABA at T24, suggesting neurotoxicity. GABA is an inhibitor neurotransmitter which can regulate  
451 dopamine neurotransmission (Bak et al., 2006). In *Corbicula fluminea* 0.5  $\mu\text{g L}^{-1}$  CBZ 30d modulated 3  
452 genes coding for one GABA transporter and two GABA receptors in gonads, the mantle and digestive  
453 glands (Chen et al., 2021). Hg is known to alter the regulation of GABA amount in vertebrates  
454 (Fitsanakis and Aschner, 2005). GABA is involved in reproduction, notably steroidogenesis (Maguire  
455 et al., 2005) and spermatogenesis in scallops (Watanabe et al., 2014; Nagasawa et al., 2015). In  
456 *Mytilus edulis*, analyses along seasons showed that GABA was linked to the gonadal development  
457 (Kronberg et al., 2021). Similarly, the co-exposure might cause reprotoxicity in *D. polymorpha*: 5  $\mu\text{g L}^{-1}$   
458 CBZ 15 weeks delayed spermatogenesis (Magniez et al., 2018). Nonetheless, here no DAP was  
459 involved in spermatogenesis. As such, data underlined the need for more studies targeting gonads.

460

#### 461 4.2. Common molecular toxicity pathways

462 CBZ and MeHg single and co-exposures did not significantly modulate antioxidant system. MeHg  
463 and the co-exposure increased 1.6x the abundance of GST2 at the proteome level at T24, without  
464 significant modulation in GST enzyme activity level. This suggested ROS production, as expected for  
465 MeHg and CBZ exposure, and that observed increase in protein abundance likely happened to  
466 maintain cellular functions. In *Chlamys farreri* exposure to 50  $\text{ng L}^{-1}$  IHg 96h decreased 1.2x CAT and  
467 1.5x GPx activities in digestive glands but did not cause a lipid peroxidation (Zhang et al., 2010). In  
468 the same line, in *Cerastoderma edule*, exposure to 5  $\mu\text{g L}^{-1}$  CBZ 48h increased 2x the ROS production  
469 ( $\text{H}_2\text{O}_2$ ) without significant change in SOD, CAT and GST activities (Jaouani et al., 2022). In copepods

470 *Tigriopus japonicus* and *Paracyclina nana*, exposure to 1 – 1000 ng L<sup>-1</sup> MeHg 24h increased 1.2 to 1.4x  
471 ROS levels and modulated the phosphorylation status of mitogen activated protein kinase (MAPK)  
472 pathways (1.2 to 1.5x; Lee et al., 2017a, 2017b). Data globally supported an efficient basal level of  
473 antioxidant defenses in *D. polymorpha*. Unchanged antioxidant activities and LOOH abundance  
474 suggested that *D. polymorpha* tolerated our experimental conditions. In the same line, single and co-  
475 exposure unchanged mt gene expression level. In *Caenorhabditis elegans*, exposure to 60 mg L<sup>-1</sup>  
476 MeHg 15h did not modulate mt gene expression, but a knockout mutant of mt-gene showed a higher  
477 sensitivity to MeHg (Helmcke and Aschner, 2010), supporting that background MT was instrumental  
478 for defense against MeHg. This could also be the case in *D. polymorpha*, here.

479 Single and co-exposures at T24 also caused a modulation of DAPs involved in protein turnover  
480 (e.g. transcription, translation) and amino acid metabolism, while CBZ at T24 and the co-exposure at  
481 both timepoints caused an alteration of proteinogenic amino acids. Similarly, in digestive glands of  
482 *M. galloprovincialis*, 80 ng L<sup>-1</sup> and 8 µg L<sup>-1</sup> CBZ 3d modulated proteins involved in amino acid  
483 metabolism and transcription/translation processes (Dumas et al., 2022b). Authors hypothesized an  
484 increase of protein biosynthesis due to a cellular stress (Dumas et al., 2022b). In adductor muscles of  
485 *Ruditapes philippinarum*, 20 µg L<sup>-1</sup> IHg 48h caused a reduction of alanine, arginine, branched-chain  
486 amino acids, glutamine, glutamate, glycine compared to Ctl (Liu et al., 2011). Globally, data  
487 suggested here that both CBZ and MeHg increased transcription and translation, most likely to fight  
488 against potential toxicity resulting from their bioaccumulation.

489 In aquatic invertebrates, amino acid consumption can produce energy in response to stress  
490 through an alternative pathway (Sokolova et al., 2012). Here single and co-exposures altered energy  
491 metabolism through amino acid, lipid and sugar consumption. CBZ, MeHg and the co-exposure at T24  
492 caused a modulation of DAPs involved in the energy metabolism (e.g. “glucose homeostasis”,  
493 “glycerolipid metabolism”). In addition, the co-exposure resulted in a depletion of fumaric acid and  
494 glyceric acid at T24, and an accumulation of glycerolipids (glycerol, glycerol-3-phosphate) and lactic

495 acid at T72. Fumaric acid is an important metabolite of TCA cycle; glyceric acid is involved in  
496 glycerolipid metabolism; and lactic acid is an end product of anaerobic pyruvate metabolism of  
497 glucose (De Zwaan and Dando, 1984). In the same line, “HIF signaling pathway” was enriched by  
498 MeHg single exposure, although oxygen saturation was stable in all exposures. HIF is a transcription  
499 factor maintaining cellular homeostasis in aquatic invertebrates in suboxic conditions (Giannetto et  
500 al., 2015). Similarly, in gills of *Mytilus galloprovincialis* in a Hg-polluted site in the Mediterranean Sea,  
501 hif- $\alpha$  gene expression was up-regulated 2.3x in comparison with a reference site (Maisano et al.,  
502 2017). In astrocytes of rat, 2 mg L<sup>-1</sup> MeHg 30 min down-regulated 2.2x the expression of hif-1 $\alpha$ ,  
503 together with a 1.5x decrease of cell proliferation (Chang et al., 2019). Here, shift from aerobic to  
504 anaerobic metabolism through HIF 1 signaling pathway in *D. polymorpha* most likely prevented  
505 cytotoxic effect of MeHg, that would have caused metabolome alteration that are not observed here.  
506 Thus, data here suggested a switch from TCA cycle to an anaerobic metabolism, particularly in the co-  
507 exposure. Similarly, in adductor muscles of *R. philippinarum*, exposure to 20  $\mu\text{g L}^{-1}$  IHg 48h caused an  
508 accumulation of lactate and succinate, and a depletion of acetoacetate and ATP vs Ctl (Liu et al.,  
509 2011).

510 Data here supported that in *D. polymorpha* the co-exposure triggered more alterations than  
511 single exposures at concentrations representative of contaminated areas, although the zebra mussel  
512 appears to tolerate these experimental conditions. As bioaccumulation did not seem to be correlated  
513 to the number of molecular modulations, this is attributed to the combined molecular toxicity  
514 mechanisms of both pollutants. Similarly, in *D. polymorpha*, the co-exposure to 3.9  $\mu\text{g L}^{-1}$  CBZ + 280  
515 ng L<sup>-1</sup> MeHg resulted in no significant modulation at T24 in single and the co-exposure, while 7d co-  
516 exposure caused a significant decrease of 25 metabolites involved in antioxidant, amino acid and  
517 energy metabolism (e.g. GSH metabolism, glycine, serine and threonine metabolism, galactose  
518 metabolism) (Baratange et al., 2022).

519

## 520 **5. Conclusion**

521 The present work combined several approaches at different biological organization levels to  
522 identify molecular toxicity pathways of CBZ and MeHg in *D. polymorpha* (figure 6). Alterations of  
523 amino acid metabolism, energy metabolism, development, and stress responses were common to  
524 the three exposures. In line with the initial hypothesis, the co-exposure caused more alterations than  
525 single exposures in *D. polymorpha*. Nonetheless, data showed a low-toxicity level, supporting that  
526 exposures were well tolerated by *D. polymorpha*. Untargeted proteogenomics identified proteins  
527 involved “calcium signaling pathway” for CBZ exposure, “HIF-1”, and “Toll and Imd signaling  
528 pathways” for MeHg exposure and “dopaminergic synapse” for the co-exposure. These specific  
529 responses underlined the challenge to predict effects of a co-exposure based on responses observed  
530 in single exposures. As such, it is a priority to consider pollutant cocktails in future research,  
531 particularly at environmental concentrations, to better understand and anticipate the ecotoxicity of  
532 pollutants in more complex and realistic scenarios.

533

## 534 **Funding**

535 This research was funded by Grand Reims through the Aquasurv Chair, by the French National  
536 program EC2CO (Ecosphere Continentale et Cotiere) through the CARMA n°12837 program.

537

## 538 **Declaration of competing interests**

539 The authors have no competing interests to declare.

540

## 541 **Acknowledgements**



542 We are grateful to the Mobicyte Platform at URCA and EcoChimie Platform (EcoChim) at UMS OSUR  
543 3343 for access to qPCR and metabolomics facilities, respectively and technical support. Authors  
544 would like to thank Nicolas Borie and Fanny Louis for technical help, and Maxime Leprêtre for  
545 analytical advice. Authors also benefited from the French GDR “Aquatic Ecotoxicology” framework  
546 which aims at fostering stimulating scientific discussions and collaborations for more integrative  
547 approaches.

548

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854

855 **Table 1:** List of the ten most increased and reduced proteins ( $\log_2$  fold change vs Ctl; blastp,  
856 Swissprot) in *D. polymorpha* exposed to CBZ ( $6.1 \pm 0.2 \mu\text{g L}^{-1}$ ), MeHg ( $460 \pm 20 \text{ ng L}^{-1}$ ) and the co-  
857 exposure at T24. Significant differences vs Ctl appear in bold.

Functional annotation	%sequence identity	E-value	CBZ	MeHg	CBZ + MeHg
<i>Intracellular transport</i>					
Importin-5	66.8%	$< 1\text{E}^{-199}$	1.08	0.94	<b>1.45</b>
Protein transport protein Sec31A	59.5%	$< 1\text{E}^{-199}$	0.77	<b>1.26</b>	<b>1.20</b>
<i>Response to stress</i>					
Calumenin-A-like	62.7%	$9.3\text{E}^{-139}$	0.68	1.14	<b>1.58</b>
Cathepsin L1-like	45.2%	$2.0\text{E}^{-40}$	0.26	0.68	<b>1.49</b>
Cathepsin L1 isoform X4	46.4%	$1.1\text{E}^{-75}$	<b>1.09</b>	<b>1.05</b>	<b>1.38</b>
CD109 antigen-like	46.6%	$2.8\text{E}^{-37}$	1.21	<b>1.58</b>	1.16
Cyclin-related protein FAM58A	56.1%	$1.4\text{E}^{-102}$	<b>-1.14</b>	-0.65	<b>-1.14</b>
<i>Signal transduction</i>					
Guanine nucleotide-binding protein G(S) subunit alpha	84%	$< 1\text{E}^{-199}$	1.00	1.22	<b>1.42</b>
Phosphotransferase 2.7.1	48.2%	$3.9\text{E}^{-31}$	0.49	<b>1.49</b>	0.85
Receptor protein-tyrosine kinase	35.2%	$2.1\text{E}^{-16}$	<b>1.12</b>	0.42	0.00
<i>Transcription and translation</i>					
Alanine--glyoxylate aminotransferase	65.4%	$4.2\text{E}^{-180}$	0.00	0.74	<b>1.58</b>
Cleavage and polyadenylation specificity factor subunit 2	65.9%	$< 1\text{E}^{-199}$	<b>1.32</b>	0.42	1.00
Eukaryotic translation initiation factor 3 subunit J	57.5%	$2.4\text{E}^{-84}$	<b>1.12</b>	1.00	<b>1.42</b>

Isoleucyl tRNA synthetase	48.2%	1.7E <sup>-104</sup>	1.14	<b>1.68</b>	0.68
Pre-mRNA-splicing factor 3	63.9%	< 1E <sup>-199</sup>	<b>1.68</b>	0.85	0.85
RNA-binding protein 4	67.2%	2.1E <sup>-114</sup>	<b>1.22</b>	0.74	<b>1.15</b>
SAP domain-containing protein	43.0%	7.1E <sup>-43</sup>	0.64	0.00	<b>1.47</b>
THO complex subunit 1	54.6%	< 1E <sup>-199</sup>	1.00	<b>1.32</b>	<b>1.50</b>
tRNA nucleotidyltransferase (CCA-adding enzyme)	56.6%	7.3E <sup>-165</sup>	0.70	<b>1.39</b>	<b>1.25</b>
Zinc finger ZZ-type and EF-hand domain-containing protein 1	56.7%	< 1E <sup>-199</sup>	<b>1.14</b>	0.85	<b>1.38</b>
<i>Others</i>					
3-hydroxyisobutyrate dehydrogenase	35.9%	6.9E <sup>+00</sup>	<b>-1.51</b>	<b>-1.74</b>	-0.74
Multifunctional fusion protein	65.5%	7.1E <sup>-114</sup>	1.26	0.68	<b>1.68</b>
Por_secre_tail domain-containing protein	42.6%	< 1E <sup>-199</sup>	<b>1.38</b>	0.93	1.20
Spectrin beta chain, non-erythrocytic 5	65.0%	< 1E <sup>-199</sup>	<b>1.35</b>	<b>1.30</b>	1.06
Glutamate carboxypeptidase 2	48.4%	< 1E <sup>-199</sup>	<b>1.14</b>	0.49	0.00
<i>Uncharacterized</i>					
Uncharacterized protein LOC116288423	40.0%	4.4E <sup>-04</sup>	1.00	<b>1.86</b>	-0.68
Uncharacterized protein LOC116288423	41.5%	1.1E <sup>-04</sup>	0.42	<b>1.81</b>	-0.26

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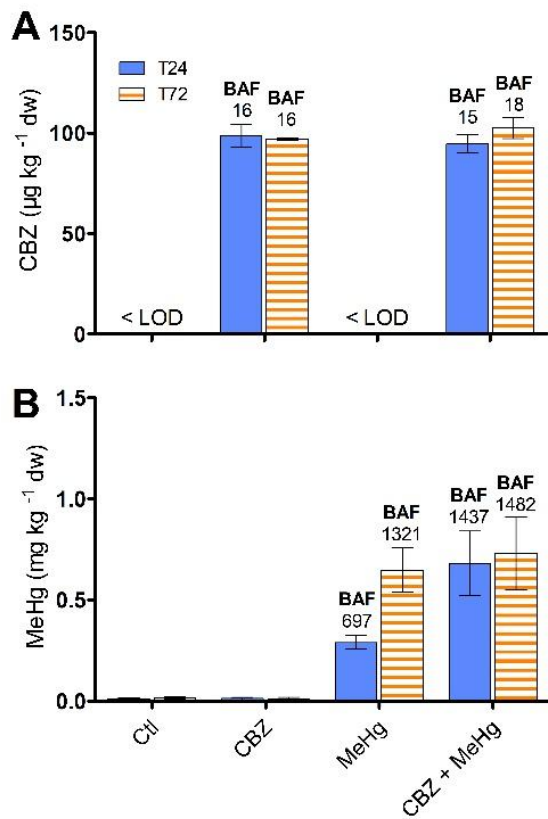
860 **Table 2:** Relative gene expression levels of *cat*, *gst*, *sod* and *mt* genes, enzyme activities of CAT (U mg  
861  $\text{prot}^{-1}$ ), GST ( $\mu\text{mol CDNB g prot}^{-1} \text{ min}^{-1}$ ), SOD (U mg  $\text{prot}^{-1}$ ), abundance of MT (mg g  $\text{prot}^{-1}$ ) and LOOH  
862 ( $\mu\text{mol TBH g prot}^{-1}$ ) in gonads, digestive glands, and gills of *D. polymorpha* exposed to CBZ ( $6.1 \pm 0.2$   
863  $\mu\text{g L}^{-1}$ ), MeHg ( $460 \pm 20 \text{ ng L}^{-1}$ ), the co-exposure, and Ctl at T24 and T72 (mean $\pm$ SEM, n = 3 to 8).

	Ctl		CBZ		MeHg		CBZ+MeHg	
	T24	T72	T24	T72	T24	T72	T24	T72
<b>GONADS</b>								
<i>cat</i>	1.00 $\pm$ 0.23	1.00 $\pm$ 0.32	0.65 $\pm$ 0.14	0.65 $\pm$ 0.15	0.56 $\pm$ 0.12	0.77 $\pm$ 0.18	0.57 $\pm$ 0.13	1.20 $\pm$ 0.21
<i>gst</i>	1.00 $\pm$ 0.32	1.00 $\pm$ 0.23	1.10 $\pm$ 0.12	0.77 $\pm$ 0.08	0.71 $\pm$ 0.18	0.56 $\pm$ 0.09	0.43 $\pm$ 0.14	0.81 $\pm$ 0.14
<i>sod</i>	1.00 $\pm$ 0.12	1.00 $\pm$ 0.17	0.66 $\pm$ 0.18	1.14 $\pm$ 0.15	0.53 $\pm$ 0.15	0.85 $\pm$ 0.19	0.49 $\pm$ 0.10	1.03 $\pm$ 0.21
<i>mt</i>	1.00 $\pm$ 0.18	1.00 $\pm$ 0.32	0.86 $\pm$ 0.25	0.78 $\pm$ 0.15	0.61 $\pm$ 0.18	0.81 $\pm$ 0.16	0.43 $\pm$ 0.10	0.81 $\pm$ 0.30
CAT	55.5 $\pm$ 3.3	52.9 $\pm$ 5.1	50.2 $\pm$ 6.2	60.5 $\pm$ 5.4	59.3 $\pm$ 5.0	50.8 $\pm$ 3.2	72.5 $\pm$ 2.8	51.1 $\pm$ 5.1
GST	115 $\pm$ 8	168 $\pm$ 10	135 $\pm$ 18	173 $\pm$ 15	124 $\pm$ 8	190 $\pm$ 23	140 $\pm$ 20	138 $\pm$ 25
SOD	4.10 $\pm$ 0.31	2.89 $\pm$ 0.53	3.58 $\pm$ 0.32	2.90 $\pm$ 0.40	3.78 $\pm$ 0.17	2.66 $\pm$ 0.70	3.78 $\pm$ 0.14	2.57 $\pm$ 0.58
MT	9.07 $\pm$ 0.21	8.73 $\pm$ 0.24	10.6 $\pm$ 0.8	10.5 $\pm$ 0.7	10.1 $\pm$ 0.4	8.97 $\pm$ 0.35	10.5 $\pm$ 0.4	7.50 $\pm$ 0.34
LOOH	40.6 $\pm$ 4.6	26.7 $\pm$ 2.1	43.2 $\pm$ 6.0	28.7 $\pm$ 2.0	44.6 $\pm$ 2.4	26.7 $\pm$ 1.8	36.5 $\pm$ 3.3	31.8 $\pm$ 2.4
<b>DIGESTIVE GLANDS</b>								
<i>cat</i>	1.00 $\pm$ 0.17	1.00 $\pm$ 0.19	0.73 $\pm$ 0.10	1.31 $\pm$ 0.12	0.77 $\pm$ 0.07	1.31 $\pm$ 0.18	0.71 $\pm$ 0.08	1.26 $\pm$ 0.22
<i>gst</i>	1.00 $\pm$ 0.22	1.00 $\pm$ 0.22	0.72 $\pm$ 0.14	1.44 $\pm$ 0.34	0.75 $\pm$ 0.17	1.08 $\pm$ 0.32	0.70 $\pm$ 0.10	1.50 $\pm$ 0.31
<i>sod</i>	1.00 $\pm$ 0.12	1.00 $\pm$ 0.26	0.54 $\pm$ 0.15	1.11 $\pm$ 0.18	0.84 $\pm$ 0.17	1.35 $\pm$ 0.25	0.87 $\pm$ 0.10	0.95 $\pm$ 0.19
<i>mt</i>	1.00 $\pm$ 0.17	1.00 $\pm$ 0.40	0.93 $\pm$ 0.18	1.09 $\pm$ 0.32	0.92 $\pm$ 0.26	1.25 $\pm$ 0.12	0.86 $\pm$ 0.18	0.78 $\pm$ 0.17
CAT	130 $\pm$ 10	160 $\pm$ 9	127 $\pm$ 10	173 $\pm$ 11	130 $\pm$ 7	149 $\pm$ 8	113 $\pm$ 15	204 $\pm$ 25
GST	185 $\pm$ 15	178 $\pm$ 13	195 $\pm$ 19	179 $\pm$ 25	207 $\pm$ 28	192 $\pm$ 14	178 $\pm$ 36	213 $\pm$ 28
SOD	2.39 $\pm$ 0.11	2.39 $\pm$ 0.17	2.40 $\pm$ 0.14	2.45 $\pm$ 0.10	2.62 $\pm$ 0.14	2.26 $\pm$ 0.15	2.55 $\pm$ 0.22	2.89 $\pm$ 0.13
MT	16.4 $\pm$ 0.8	15.4 $\pm$ 0.3	17.2 $\pm$ 0.5	15.9 $\pm$ 0.5	16.8 $\pm$ 0.3	15.3 $\pm$ 0.9	19.6 $\pm$ 0.5	18.1 $\pm$ 0.6

LOOH	55.6±8.0	42.5±3.5	48.7±3.3	43.4±2.0	52.8±4.6	57.6±7.4	54.1±4.8	52.7±7.1
<b>GILLS</b>								
cat	1.00±0.12	1.00±0.22	1.27±0.26	1.52±0.26	0.86±0.18	1.14±0.11	1.68±0.24	1.15±0.13
gst	1.00±0.14	1.00±0.07	0.99±0.07	0.74±0.09	0.97±0.05	1.54±0.29	1.21±0.14	1.17±0.16
sod	1.00±0.11	1.00±0.23	1.33±0.16	1.32±0.31	0.90±0.14	1.23±0.57	1.67±0.22	1.57±0.39
mt	1.00±0.09	1.00±0.29	1.04±0.26	0.86±0.12	0.60±0.12	1.28±0.27	0.95±0.27	1.01±0.23
CAT	23.3±0.9	23.1±1.7	25.6±1.7	24.0±2.2	25.9±1.2	22.9±1.5	24.0±1.2	26.0±2.5
GST	130±11	105±4	106±9	106±11	139±6	109±8	128±9	112±8
SOD	2.34±0.05	2.40±0.11	2.34±0.16	2.21±0.15	2.50±0.10	2.12±0.06	2.21±0.06	2.30±0.09
MT	13.3±1.4	16.5±0.6	12.0±0.5	13.4±0.5	15.5±1.4	13.4±0.5	17.0±2.3	14.9±0.9
LOOH	28.9±4.0	31.2±5.6	40.9±5.6	27.5±5.0	34.1±5.3	33.7±2.3	27.8±3.6	29.8±3.3

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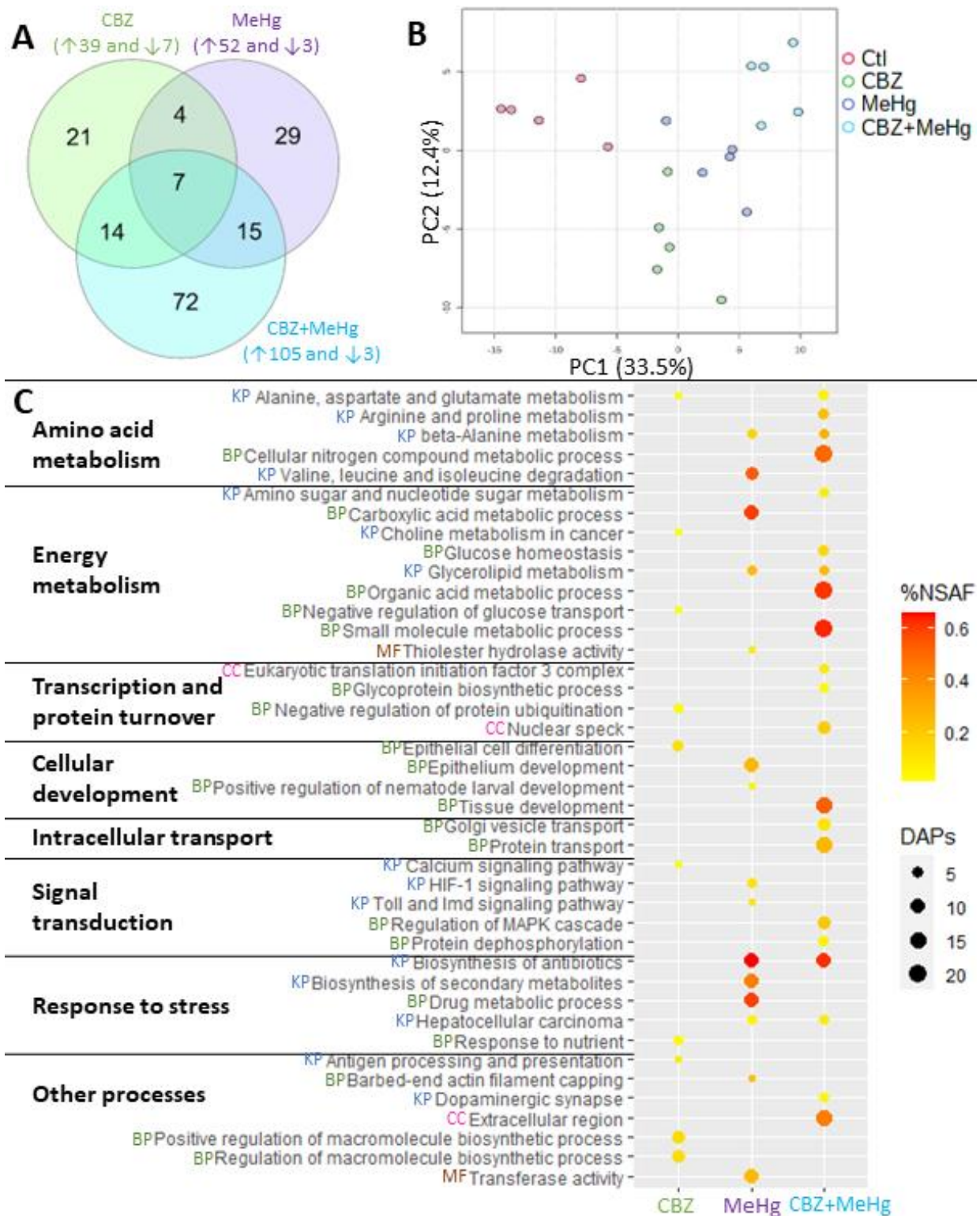


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868

869 **Figure 1:** Bioaccumulation (mean  $\pm$  SEM, n = 3) of **(A)** CBZ and **(B)** MeHg in *D. polymorpha* exposed to870 CBZ ( $6.1 \pm 0.2 \mu\text{g L}^{-1}$ ), MeHg ( $460 \pm 20 \text{ ng L}^{-1}$ ), the co-exposure (CBZ+MeHg) and Ctl. BAF ( $\text{L kg}^{-1}$ ):

871 bioaccumulation factor



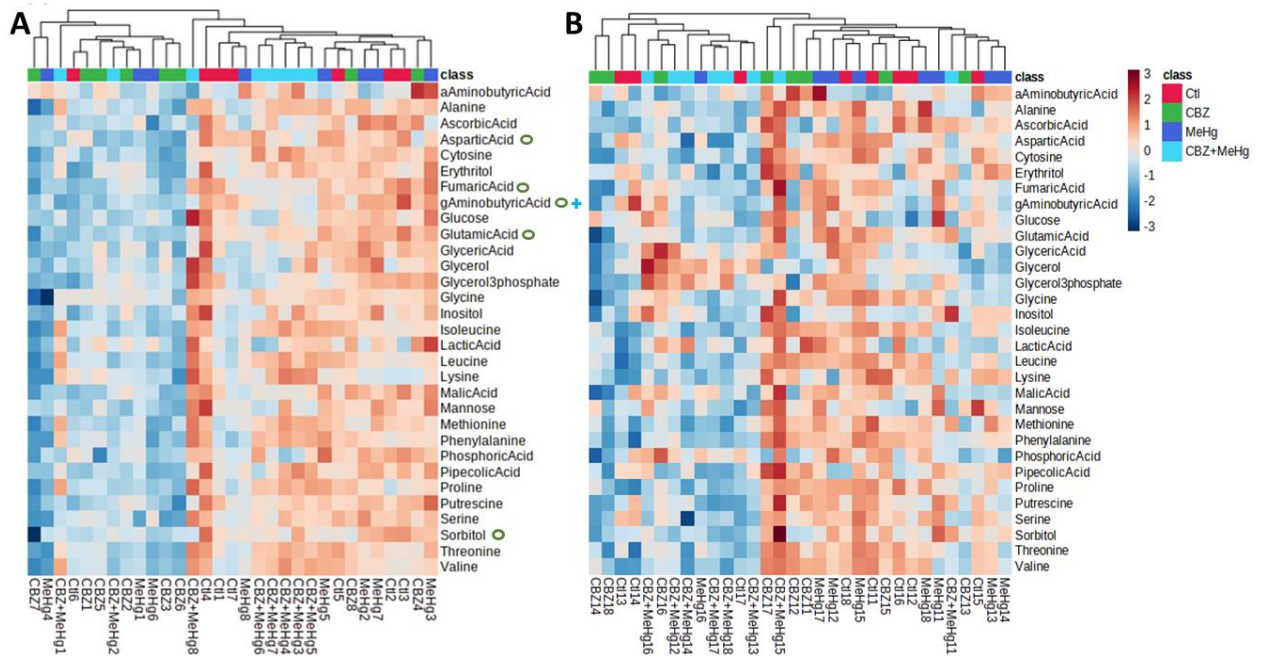
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873 **Figure 2: (A)** Venn diagram of differentially abundant proteins, **(B)** PCA of normalized spectral count

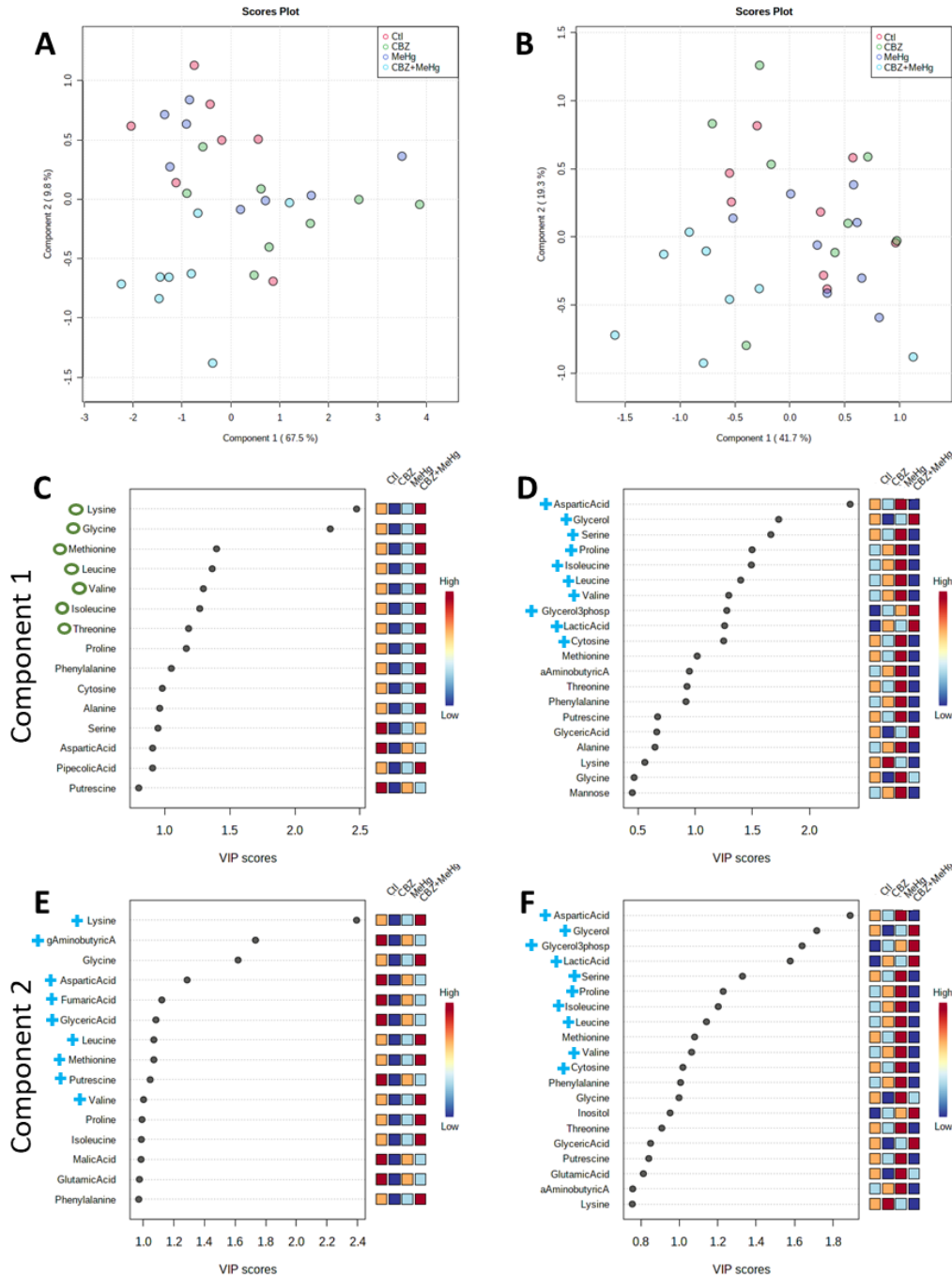
874 data of DAPs vs the mean of Ctl, and **(C)** enriched KEGG Pathways (KPs, blue), biological

875 processes (BPs, green), molecular functions (MFs, brown) and cellular components (CC, pink)

876 GO-terms in *D. polymorpha* exposed to CBZ ( $6.1 \pm 0.2 \mu\text{g L}^{-1}$ , green), MeHg ( $460 \pm 20 \text{ ng L}^{-1}$ ,  
 877 purple), the co-exposure (CBZ+MeHg, cyan) and Ctl (red) at T24.

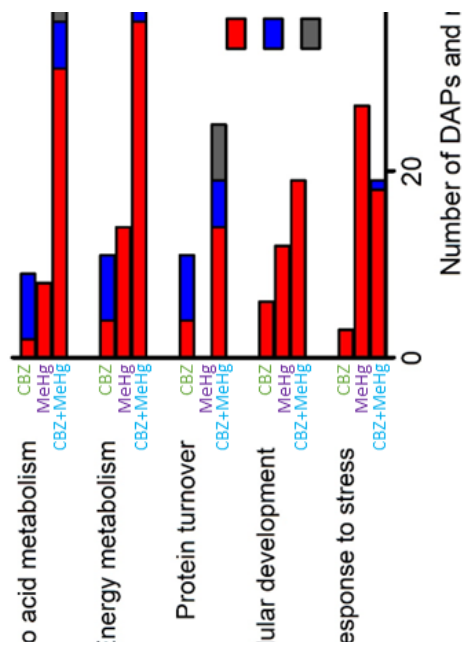


878  
 879 **Figure 3:** Heatmaps of the 31 quantified metabolites at T24 (A) and T72 (B) in *D. polymorpha* exposed  
 880 to CBZ ( $6.1 \pm 0.2 \mu\text{g L}^{-1}$ ; green), MeHg ( $460 \pm 20 \text{ ng L}^{-1}$ ; purple), the co-exposure (CBZ+MeHg; cyan),  
 881 and Ctl (red) (n = 7 to 8). Modulated metabolites by CBZ (green circle) and the co-exposure (cyan  
 882 cross) were identified by ANOVA analyses ( $p < 0.05$  and  $FC > 1.4$ ).



883

884 **Figure 4:** PLS-DAs (**A, B**) and VIP scores (**C to F**) of the 31 quantified metabolites at T24 (**A, C, E**) and  
 885 T72 (**B, D, F**) in *D. polymorpha* exposed to CBZ ( $6.1 \pm 0.2 \mu\text{g L}^{-1}$ ; green), MeHg ( $460 \pm 20 \text{ng L}^{-1}$ ;  
 886 purple), the co-exposure (CBZ+MeHg, cyan), and Ctl (red) ( $n = 7$  to  $8$ ). Modulated metabolites  
 887 in CBZ (green circle) and the co-exposure (cyan cross) were identified by multivariate  
 888 analyses ( $\text{VIP} > 1$  and  $\text{FC} > 1.4$ ).



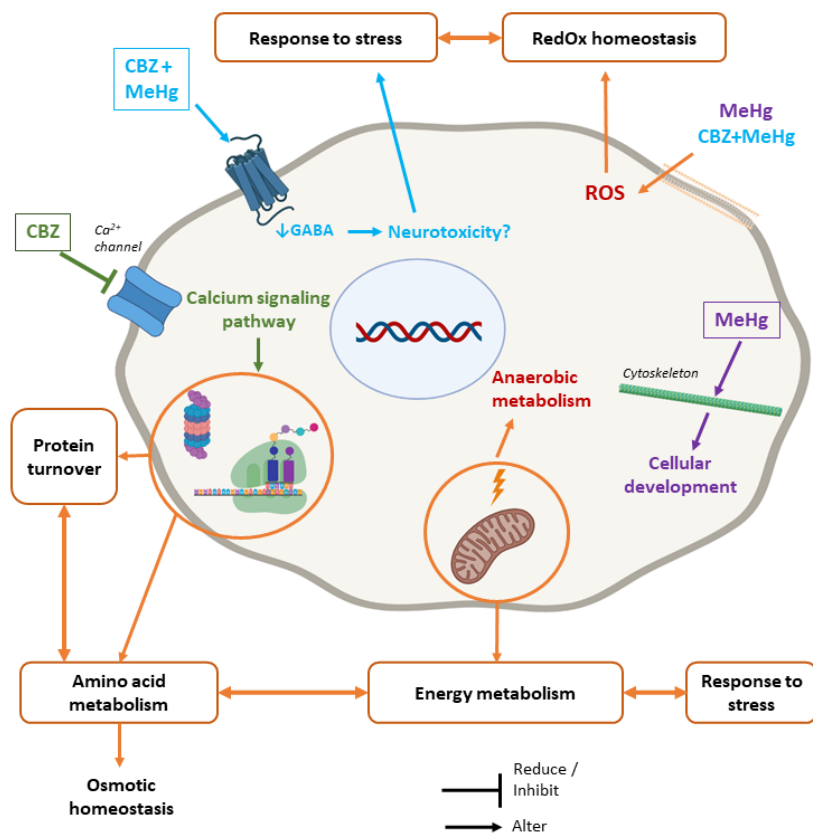
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890 **Figure 5:** Number of significant modulations observed at proteome and metabolome levels in *D.*

891 *polymorpha* exposed to CBZ ( $6.1 \pm 0.2 \mu\text{g L}^{-1}$ ; green), MeHg ( $460 \pm 20 \text{ ng L}^{-1}$ ; purple) and the

892 co-exposure (CBZ+MeHg, cyan) at T24 and T72. DAP = differentially abundant protein.

893



894

895 **Figure 6:** Main molecular responses observed in *D. polymorpha* exposed to CBZ ( $6.1 \pm 0.2 \mu\text{g L}^{-1}$ ;  
 896 green), MeHg ( $460 \pm 20 \text{ ng L}^{-1}$ ; purple), the co-exposure (cyan) and subsequent hypotheses.  
 897 Arrows in orange show common molecular toxicity pathways of single and co-exposures. ROS  
 898 = reactive oxygen species, GABA =  $\gamma$ -aminobutyric acid (designed with BioRender).