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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-04161067>

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 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; CH_3HgII) 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 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 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 ± 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 μm , 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 $-80\text{ }^\circ\text{C}$ 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 $-20\text{ }^\circ\text{C}$. For
143 qPCR and biochemical analyses, 16 mussels were dissected, and digestive glands, gills and gonads
144 frozen in liquid nitrogen and stored at $-80\text{ }^\circ\text{C}$.

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⁻¹) with orthophosphoric acid (0.1%) (B)
155 was used on a reverse-phase Agilent Pursuit XRs 5 C₁₈ 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⁻¹ 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⁻¹ CBZ, and by adding an internal
165 standard of 50 ng mL⁻¹ CBZ-d₁₀ 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 HNO₃ (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 μ 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 μ 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 μ L out of 50 μ 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 μ m, 100 Å,
192 75 μ m id x 50 cm) at a flow rate of 0.2 μ 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 ≥ 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 μL 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 μL 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 μL 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 μL glass vials and dried for 1h at $32\text{ }^{\circ}\text{C}$ (Genevac, miVac DNA). Dry
233 residues were resuspended in 30 μL of methoxyamine hydrochloride (Sigma-Aldrich, 25 mg L^{-1}) in
234 pyridine (Fisher chemical), and automatically homogenized at $40\text{ }^{\circ}\text{C}$ for 60min, before adding 30 μL 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 μL 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 μM . 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 H₂SO₄ 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 H₂O₂ (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⁻¹.

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 MnCl₂,
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⁻¹.

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) ≥ 1.20 and ≤ 0.83 for gene expression level
305 and biochemical analyses and $FC \geq 1.40$ and ≤ 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). 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 ≥ 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 ± 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 ± 0.1 , conductivity $500 \pm 20\ \mu S\ cm^{-1}$, oxygen saturation $75.8 \pm 0.1\ \%$

321 O₂, nitrates 9 ± 1 mg L⁻¹, nitrites 0.10 ± 0.02 mg L⁻¹ and ammonium 0.04 ± 0.02 mg L⁻¹ (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 ≤ 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⁻¹ in the fish
422 *Solea senegalensis* caused an increase of up to 3x of Na⁺, K⁺-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, Ca²⁺ 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, Ca²⁺ 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⁻¹ 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⁻¹ 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 µg 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 µg 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 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 µg L⁻¹ CBZ 48h increased 2x the ROS production
469 (H₂O₂) 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⁻¹ 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⁻¹
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⁻¹ and 8 µg L⁻¹ 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⁻¹ 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- α 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⁻¹ MeHg 30 min down-regulated 2.2x the expression of hif-1 α ,
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⁻¹ 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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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 | 1.45 |
| Protein transport protein Sec31A | 59.5% | $< 1\text{E}^{-199}$ | 0.77 | 1.26 | 1.20 |
| <i>Response to stress</i> | | | | | |
| Calumenin-A-like | 62.7% | 9.3E^{-139} | 0.68 | 1.14 | 1.58 |
| Cathepsin L1-like | 45.2% | 2.0E^{-40} | 0.26 | 0.68 | 1.49 |
| Cathepsin L1 isoform X4 | 46.4% | 1.1E^{-75} | 1.09 | 1.05 | 1.38 |
| CD109 antigen-like | 46.6% | 2.8E^{-37} | 1.21 | 1.58 | 1.16 |
| Cyclin-related protein FAM58A | 56.1% | 1.4E^{-102} | -1.14 | -0.65 | -1.14 |
| <i>Signal transduction</i> | | | | | |
| Guanine nucleotide-binding protein G(S) subunit alpha | 84% | $< 1\text{E}^{-199}$ | 1.00 | 1.22 | 1.42 |
| Phosphotransferase 2.7.1 | 48.2% | 3.9E^{-31} | 0.49 | 1.49 | 0.85 |
| Receptor protein-tyrosine kinase | 35.2% | 2.1E^{-16} | 1.12 | 0.42 | 0.00 |
| <i>Transcription and translation</i> | | | | | |
| Alanine--glyoxylate aminotransferase | 65.4% | 4.2E^{-180} | 0.00 | 0.74 | 1.58 |
| Cleavage and polyadenylation specificity factor subunit 2 | 65.9% | $< 1\text{E}^{-199}$ | 1.32 | 0.42 | 1.00 |
| Eukaryotic translation initiation factor 3 subunit J | 57.5% | 2.4E^{-84} | 1.12 | 1.00 | 1.42 |

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

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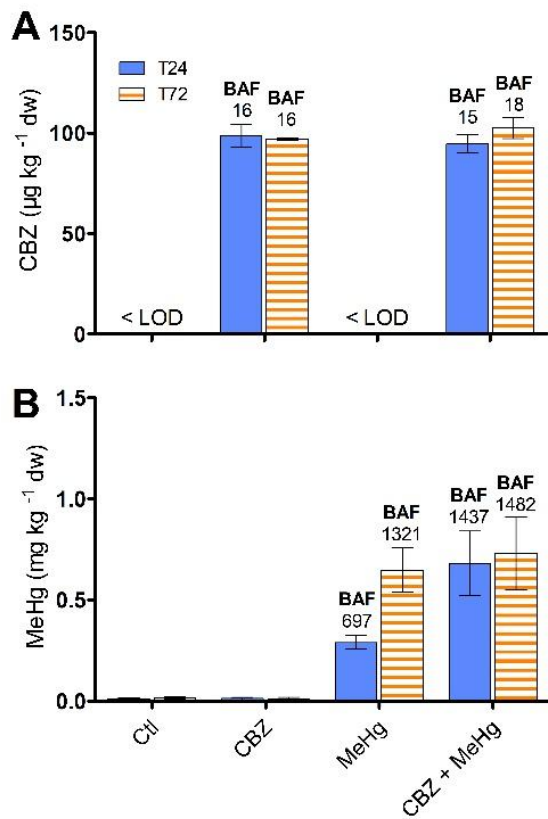
860 **Table 2:** Relative gene expression levels of *cat*, *gst*, *sod* and *mt* genes, enzyme activities of CAT (U mg
861 prot^{-1}), GST ($\mu\text{mol CDNB g prot}^{-1} \text{ min}^{-1}$), SOD (U mg prot^{-1}), abundance of MT (mg g prot^{-1}) and LOOH
862 ($\mu\text{mol TBH g prot}^{-1}$) in gonads, digestive glands, and gills of *D. polymorpha* exposed to CBZ (6.1 ± 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 |
| GONADS | | | | | | | | |
| <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 |
| DIGESTIVE GLANDS | | | | | | | | |
| <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 |
| GILLS | | | | | | | | |
| 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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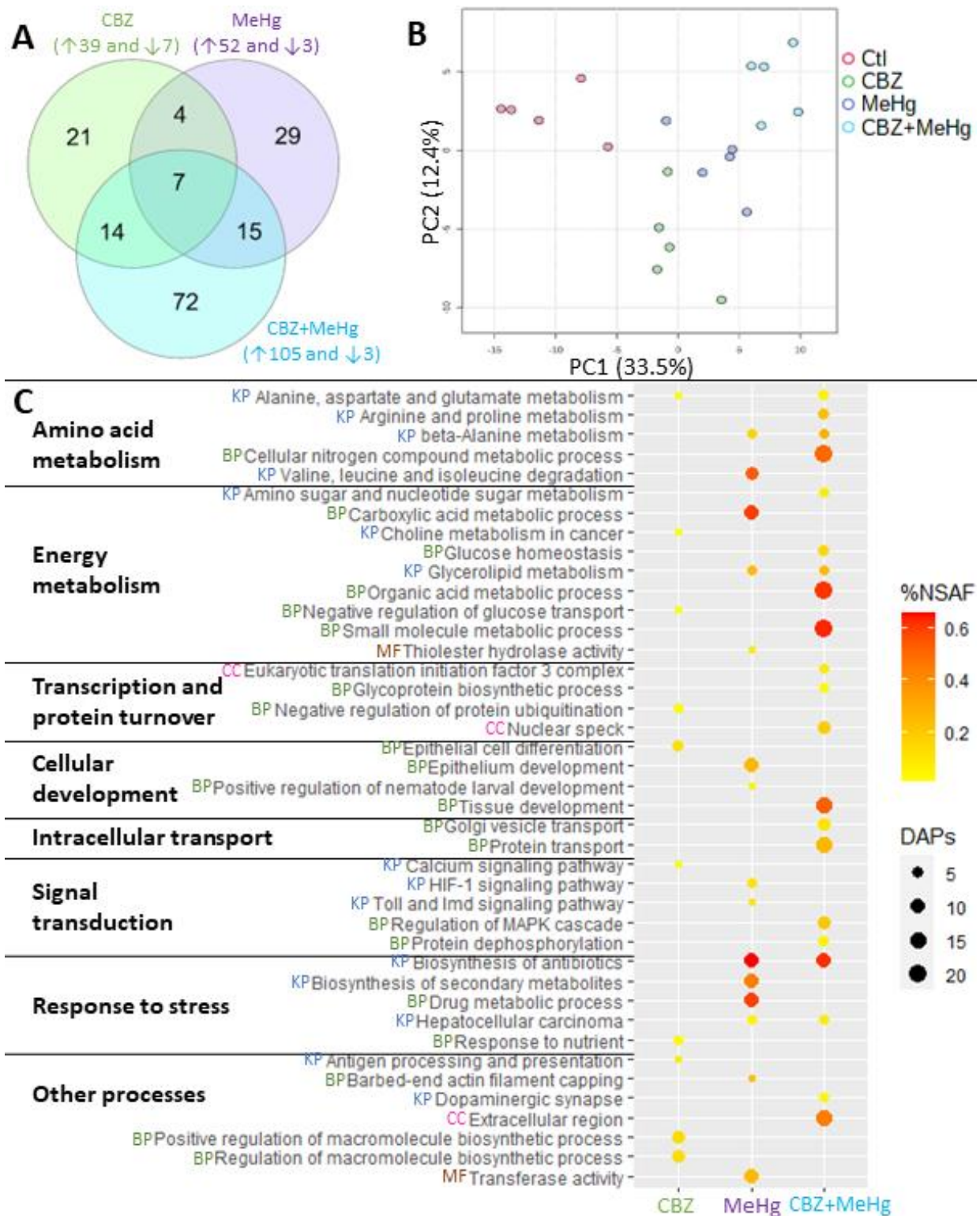


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869 **Figure 1:** Bioaccumulation (mean \pm SEM, n = 3) of **(A)** CBZ and **(B)** MeHg in *D. polymorpha* exposed to870 CBZ ($6.1 \pm 0.2 \mu\text{g L}^{-1}$), MeHg ($460 \pm 20 \text{ ng L}^{-1}$), the co-exposure (CBZ+MeHg) and Ctl. BAF (L kg^{-1}):

871 bioaccumulation factor



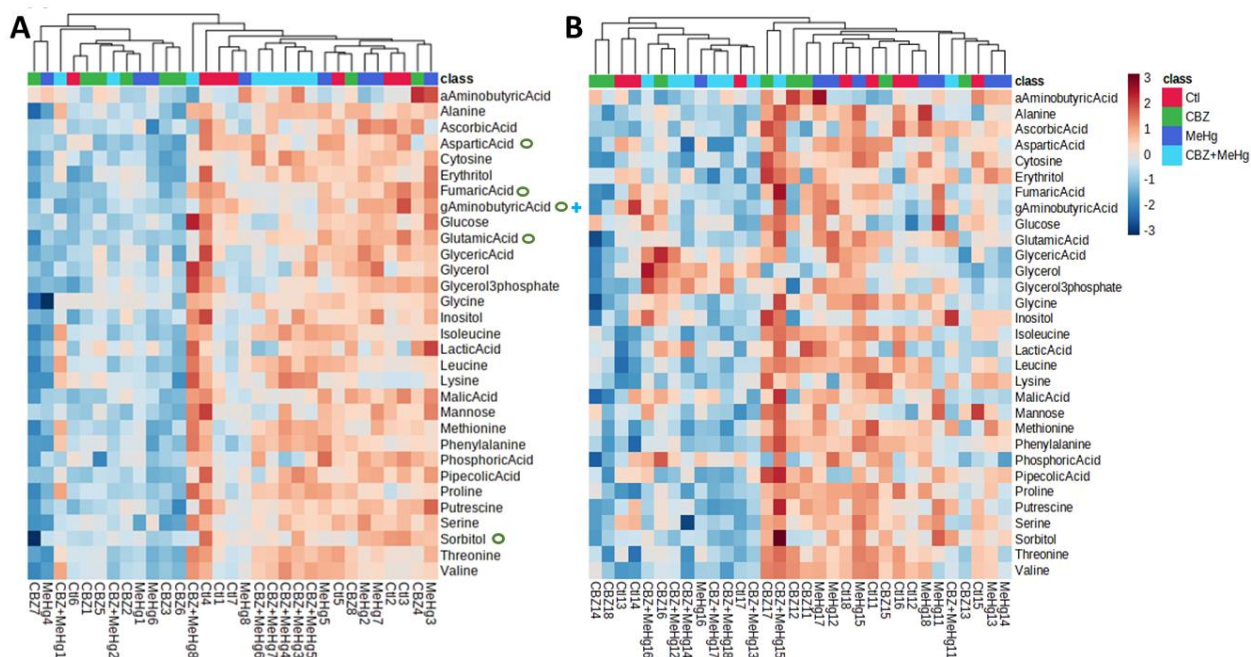
872

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

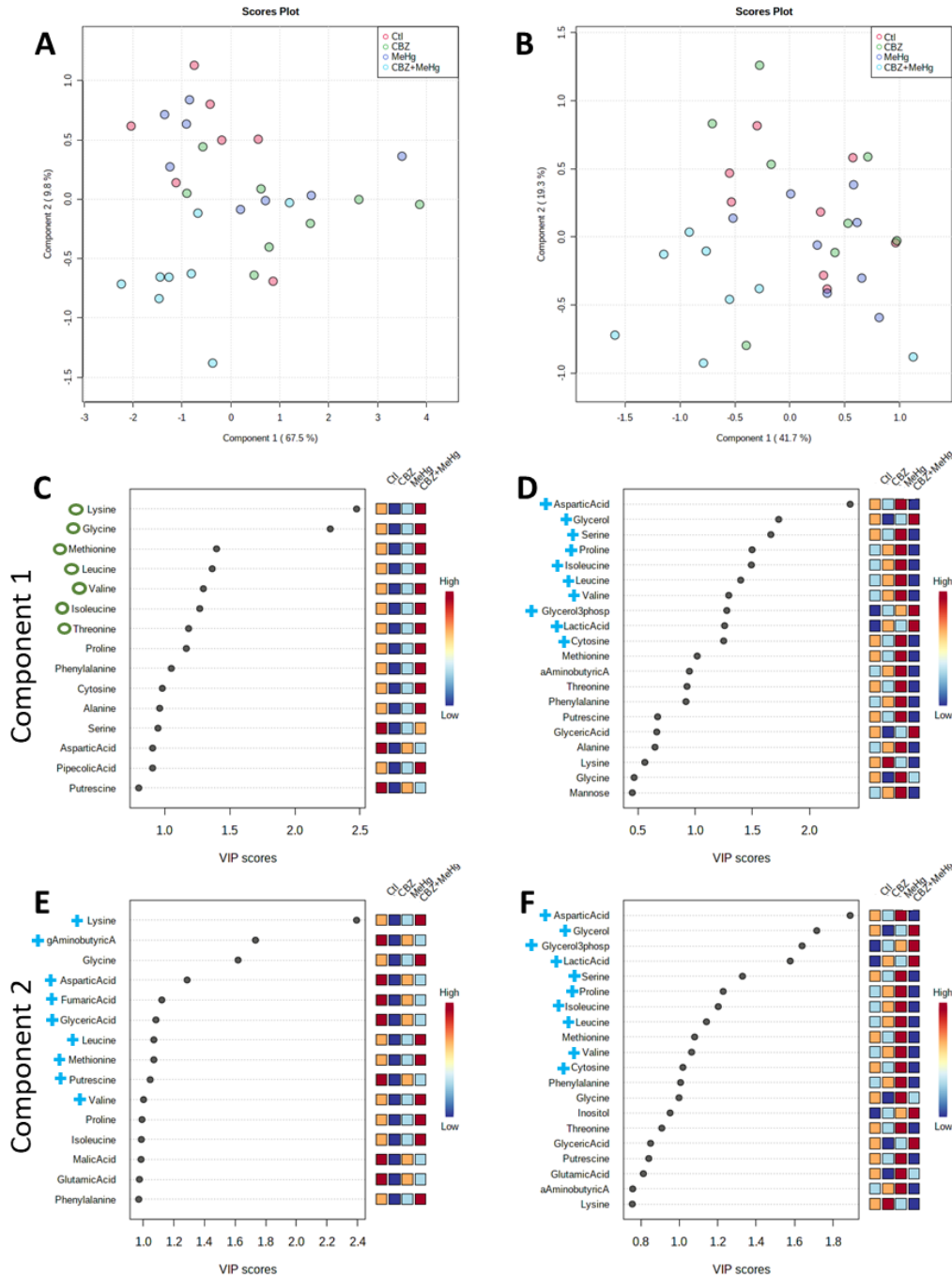
874 data of DAPs vs the mean of Ctl, and **(C)** enriched KEGG Pathways (KPs, blue), biological

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.

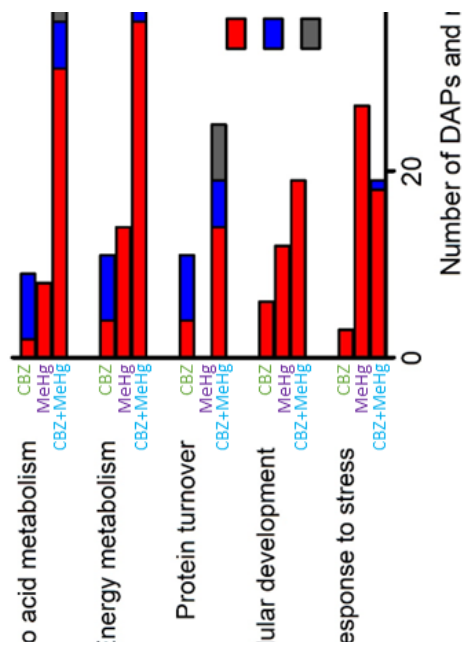


878
 879 **Figure 3:** Heatmaps of the 31 quantified metabolites at T24 (A) and T72 (B) in *D. polymorpha* exposed
 880 to CBZ ($6.1 \pm 0.2 \mu\text{g L}^{-1}$; green), MeHg ($460 \pm 20 \text{ ng L}^{-1}$; purple), the co-exposure (CBZ+MeHg; cyan),
 881 and Ctl (red) (n = 7 to 8). Modulated metabolites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



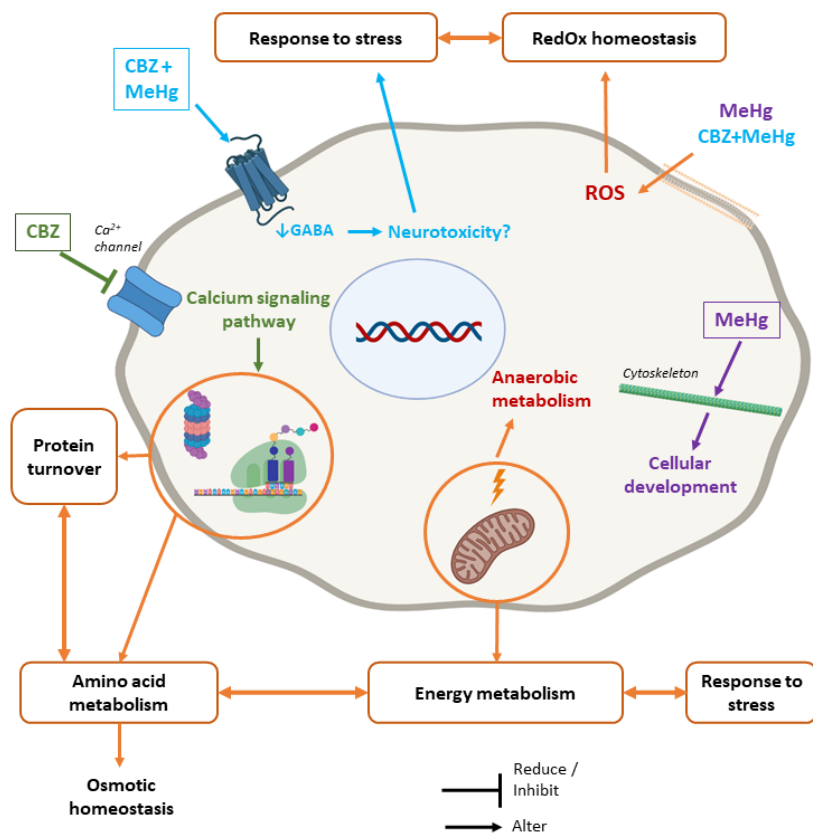
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890 **Figure 5:** Number of significant modulations observed at proteome and metabolome levels in *D.*

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 = γ -aminobutyric acid (designed with BioRender).