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1 Mixture effects of pharmaceuticals carbamazepine, diclofenac and venlafaxine on
2 *Mytilus galloprovincialis* mussel probed by metabolomics and proteogenomics
3 combined approach

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15
16 **Abstract**

17 Exposure to single molecules under laboratory conditions has led to a better understanding of the
18 mechanisms of action (MeOAs) and effects of pharmaceutical active compounds (PhACs) on non-target
19 organisms. However, not taking the co-occurrence of contaminants in the environment and their
20 possible interactions into account may lead to underestimation of their impacts. In this study, we
21 combined untargeted metabolomics and proteogenomics approaches to assess the mixture effects of
22 diclofenac, carbamazepine and venlafaxine on marine mussels (*Mytilus galloprovincialis*). Our multi-
23 omics approach and data fusion strategy highlighted how such xenobiotic cocktails induce important
24 cellular changes that can be harmful to marine bivalves. This response is mainly characterised by
25 energy metabolism disruption, fatty acid degradation, protein synthesis and degradation, and the
26 induction of endoplasmic reticulum stress and oxidative stress. The known MeOAs and molecular
27 signatures of PhACs were taken into consideration to gain insight into the mixture effects, thereby
28 revealing a potential additive effect. Multi-omics approaches on mussels as sentinels offer a
29 comprehensive overview of molecular and cellular responses triggered by exposure to contaminant
30 mixtures, even at environmental concentrations.

32 **Keywords:** multi-omics; proteomics; pharmaceutical active compounds; bivalve mollusks; data
33 fusion; adverse effects

34

35 1. Introduction

36 Pharmaceutical active compounds (PhACs) include a vast diversity of molecules, thousands of
37 which are marketed worldwide. However, it is now recognized that large consumption and release of
38 these compounds can potentially pose a threat to aquatic environments, including marine ecosystems
39 (Fabbri and Franzellitti, 2016; Mezzelani et al., 2018; Santos et al., 2010). Commonly prescribed PhACs
40 are consequently often detected in the marine environment including the antiepileptic carbamazepine
41 (CBZ), the nonsteroidal anti-inflammatory drug (NSAID) diclofenac (DCF) and the antidepressant
42 venlafaxine (VLF) (Arpin-Pont et al., 2016; Branchet et al., 2021; Desbiolles et al., 2018; Fernández-
43 Rubio et al., 2019; Moreno-González et al., 2016; Sousa et al., 2020). For instance, Sousa et al. (2020)
44 detected DCF in 20 out of 36 estuarine water samples, which is the highest frequency among the other
45 targeted PhACs. Other authors showed in their study a highest detection frequency for VLF found in
46 59% of coastal water samples (Fernández-Rubio et al., 2019). In addition, CBZ is known for its low
47 removal in wastewater treatment plants (Margot et al., 2015) and its low (bio)degradability in the
48 environment (Benotti and Brownawell, 2009; Calisto et al., 2011). For these reasons, CBZ has been
49 proposed as a possible anthropogenic marker of urban pollution in the aquatic systems (Clara et al.,
50 2004; Hai et al., 2018). Their concentration in marine ecosystems ranges from <1 to 1410 ng/L for CBZ
51 (Almeida et al., 2021), from <1 to hundreds ng/L for DCF (Bonnefille et al., 2018b) and, from <1 to few
52 hundreds ng/L for VLF (see references in Ramirez et al., 2022). These PhACs have also been measured
53 in the tissues of filter feeders, such as bivalves, at few ng/g dry weight, up to >10 ng/g (Almeida et al.,
54 2021; Alvarez-Muñoz et al., 2015; Bonnefille et al., 2018b; Martínez Bueno et al., 2013, 2014). In
55 addition, metabolites of these PhACs have been quantified after the exposure of bivalves to the parent
56 compounds in laboratory studies (Ariza-Castro et al., 2021; Boillot et al., 2015; Bonnefille et al., 2017;

57 Gomez et al., 2021), thus indicating the ability of these organisms to metabolize them. This situation
58 raises the question of the effects that PhACs may have on these non-target organisms.

59 Research has shown that PhACs can affect non-target organisms via the mechanism(s) of action
60 (MeOA) for which they were designed, as many biological targets are conserved across organisms. For
61 instance, some carbamazepine (CBZ) MeOAs known in humans have also been reported in marine
62 bivalves: CBZ leads to a reduction in intracellular cAMP levels by interacting with the adenylyl cyclase
63 system (Martin-Diaz et al., 2009), has a potential gamma-aminobutyric acid (GABA) receptor agonist
64 role (Almeida et al., 2018), interacts with voltage-dependent Na⁺ and K⁺ channels (Freitas et al., 2016)
65 and, may also induce autophagy through either endoplasmic reticulum (ER) stress induction or inositol
66 monophosphatase inhibition (Dumas et al., 2022a). For diclofenac (DCF), its main MeOA in humans is
67 cyclooxygenase COX-1 and COX-2 inhibition, which decreases prostaglandin E2 (PGE2) and
68 thromboxane production. This MeOA seems to be conserved in marine mussels (*Mytilus*
69 *galloprovincialis*), as suggested by a diminution in PGE2 after DCF exposure (Courant et al., 2017).
70 Similarly, an increase of serotonin was measured in marine mussels following exposure to VLF, an
71 antidepressant classified as a serotonin-norepinephrine reuptake inhibitor (SNRI) in humans (Ramirez
72 et al., 2022). These studies confirmed that PhACs act on non-target organisms through conserved
73 MeOAs, although unexpected MeOAs could also be involved due to biological differences between
74 species. Marine organism exposure to such PhACs could thus elicit adverse physiological effects. For
75 instance, DCF, CBZ and VLF are known to individually trigger oxidative stress, immunotoxicity and
76 genotoxicity in marine molluscs, thereby causing a decrease in their feeding activity and impairing
77 byssus production in mussels (Almeida et al., 2020; Bonnefille et al., 2018b; Fong et al., 2015; Lacaze
78 et al., 2015). Our previous studies on each individual compound also demonstrated metabolic and
79 cellular responses triggered by exposure in *M. galloprovincialis*. DCF disturbed the metabolism of
80 tyrosine (e.g. catecholamines) and tryptophan (e.g. serotonergic related compounds), with a risk for
81 impaired reproduction and osmoregulation of mussels (Bonnefille et al., 2018a). Exposure to CBZ
82 involved a disruption of lipid and amino acid metabolisms and, an intensification of protein synthesis

83 as well as of transport and catabolism processes (Dumas et al., 2022a). These molecular signatures
84 indicate a cytotoxic effect of CBZ induced by several mechanisms. VLF triggered the modulation of
85 hundreds metabolites belonging to the metabolism of amino acids (e.g. phenylalanine, tyrosine,
86 tryptophan, arginine, etc.), purine and pyrimidine metabolisms (adenosine, cyclic AMP, thymidine,
87 etc.), as well as serotonin directly related to its MeOA, and many other metabolites involved in diverse
88 functions (Ramirez et al., 2022). Such molecular disturbances induced by VLF could have an impact on
89 the reproduction and behavior of mussels. All three PhACs thus affect marine organisms through
90 toxicological pathways that are still only partially understood on the basis of the findings of studies
91 conducted under individual laboratory exposure conditions.

92 CBZ, DCF and VLF co-occur in marine ecosystems and are also jointly detected in marine organism
93 tissues (Desbiolles et al., 2018; Moreno-González et al., 2016), but the effects of mixture of these
94 PhACs have yet to be investigated. Mixture effects in the environment (e.g. treated wastewater) can
95 be additive when PhACs act independently or through the same MeOA, or synergistic or antagonistic
96 when they interfere with each other (Backhaus, 2014; Dumas et al., 2020b; Eggen et al., 2004).
97 Examples of such mixture effects are highlighted in the review of Godoy and Kummrow (2017)
98 concerning almost 200 assessments of mixture toxicity from 65 reported studies. However, most
99 assessments of mixture effects on organisms relied solely on target-oriented effect approaches
100 without considering the mechanism of action (i.e. ecotoxicological tests such as growth, luminescence
101 or reproduction inhibition, survival/mortality, etc.). For instance, Di Nica et al. (2017) showed the clear
102 presence of synergistic effects of several binary PhAC mixtures, such as diclofenac-chlortetracycline,
103 by measuring the bioluminescence inhibition response of *Aliivibrio fischeri* marine bacteria. Although
104 those approaches are suitable for predicting and assessing the effects of PhAC mixtures through
105 concentration addition or independent action models, they provide little mechanistic information on
106 toxicity pathways and may overlook unexpected joint effects. Moreover, studies considering PhAC
107 mixture effects on marine organisms, such as filter feeders (e.g. bivalves), are still sparse (Almeida et
108 al., 2022; Cunha et al., 2023).

109 Omics approaches could help shed light on a broad range of molecular changes triggered by such
110 exposures, especially at the transcriptome, proteome and metabolome levels (Martins et al., 2019).
111 Building on its potential, harnessing omics holds the key to unlocking deeper insights into the intricate
112 molecular mechanics of PhAC mixtures (Kidd et al., 2023). In the last two decades, environmental
113 metabolomics has proven effective in ecotoxicology research (Castaño-Ortiz et al., 2023; Chan et al.,
114 2023; De Marco et al., 2023; Labine et al., 2023), with advances achieved in assessing contaminant
115 MeOAs, especially when combined with other omics approaches such as proteomics (recently
116 reviewed in Dumas et al., 2022b). Multi-omics offer a key tool for in-depth exploration of molecular
117 processes involved in responses to contaminant exposure. Furthermore, to be able to take full
118 advantage of multi-omics approaches, it is essential to implement an adapted data fusion strategy in
119 order to highlight correlated signatures between different omics levels (e.g. metabolites and proteins).
120 Such a strategy enables the integrative analysis of different omics dataset despite a certain degree of
121 heterogeneity between large-scaled approaches and the intrinsic complexity of the generated
122 datasets. In our previous study, we successfully applied a mid-level data fusion strategy combining
123 metabolomics and proteogenomics data (detailed in Dumas et al., 2022a). The complementarity
124 between these two omics levels enhances confidence in the metabolic pathways and cellular processes
125 affected by contaminants, which is a major beneficial feature of this strategy. For instance, proteomics
126 provides access to functional information on proteins, which may allow us to put forward hypotheses
127 on the affected molecular and cellular processes, while the detection of modulated metabolite end
128 products of biochemical reactions could support these hypotheses if they are directly or indirectly
129 related to these processes. To explore mixture effects at the MeOAs level, it is worthwhile to harness
130 the complementarity of omics through their combination. To date, only a few multi-omics approaches
131 have been applied to study mixture effects on aquatic organisms (Dondero et al., 2010; Song et al.,
132 2016; Vandenbrouck et al., 2009).

133 The present study was designed to investigate the mixture effects of CBZ, DCF and VLF in
134 Mediterranean mussels (*M. galloprovincialis*) through an integrated metabolomics and

135 proteogenomics approach based on a mid-level data fusion strategy. We focused on a mixture of
136 PhACs with different therapeutic classes, i.e. not sharing known MeOAs. We assumed that no
137 interactions would occur at the molecular targets related to their MeOAs and hence expected to
138 observe each individual PhAC signature, as noted in our previous studies (DCF: Bonnefille et al., 2018a;
139 CBZ: Dumas et al., 2022a; VLF: Ramirez et al., 2022) and also reported in the literature.

140 2. Materials & Methods

141 2.1. Chemicals

142 LC-MS-grade solvents (acetonitrile, formic acid, dichloromethane, and methanol) were
143 obtained from CARLO ERBA Reagents (Val de Reuil, France). Ultrapure water was generated by a
144 Simplicity® Water Purification System associated with an LC-Pak® Polisher and a Millipak® Express 20
145 filter (0.22 µm) from Merck Millipore (Bedford, MA, USA). Pure analytical grade standards (purity >
146 95%) were purchased from the following suppliers: Sigma-Aldrich (now part of MERCK, Saint-Quentin-
147 Fallavier, France), Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Toronto Research Chemicals
148 (Toronto, ON, Canada).

149 2.2. Animals, experimental design and sample collection

150 *Mytilus galloprovincialis* mussels were provided by Les Compagnons de Maguelone mussel
151 farmers (Villeneuve-les-Maguelone, France) in February 2020. Mussels were transported in a mesh bag
152 to the laboratory within an hour at ambient outside temperature (14°C). Mussels (n = 105 individuals)
153 with a homogeneous shell size (7 cm ± 0.8) were cleaned and randomly distributed in 14 glass aquaria
154 (5 individuals per aquarium). Each aquarium contained 2 L of filtered seawater (filter GF/F Ø 100 µm)
155 provided by IFREMER (Palavas, France). Seawater was continuously aerated and renewed daily (static
156 renewal). Mussels were fed once daily with the *Tetraselmis suecica* algae (Greensea, Mèze, France) at
157 constant density (21,000 cells/mL) and we ensured that algae uptake was complete within 1 h.
158 Throughout the experiment, seawater physicochemical parameters were checked daily and

159 maintained constant: temperature ($13.8\text{ }^{\circ}\text{C} \pm 0.5$), oxygen ($10\text{ mg/L} \pm 0.3$), salinity ($36.7\text{‰} \pm 0.2$) and
160 pH (8.07 ± 0.05).

161 After a 7-day acclimatization period, 6 aquaria underwent solvent control exposure ($10\text{ }\mu\text{L/L}$ of
162 methanol, corresponding to a final dilution of 0.01‰ ; SC), 6 aquaria were exposed to the mixture (CBZ,
163 DCF and VLF) at a low nominal concentration of $0.1\text{ }\mu\text{g/L}$ (LD), and 6 aquaria were exposed to the
164 mixture at a higher nominal concentration of $10\text{ }\mu\text{g/L}$ (HD). The low concentration has been chosen in
165 order to get closer to environmental conditions and the higher concentration to 'magnify' certain
166 effects which would have escaped us at low concentration and which would give us a better
167 understanding of MeOAs and potential interactions. To quantify the PhACs exposure, the water was
168 sampled every days throughout the experiment, just after the aquaria had been spiked. The methods
169 of quantification have been published in our previous works (Bonnefille et al., 2018a; Dumas et al.,
170 2022a; Ramirez et al., 2022). The concentrations measured were as follows: DCF = $0.139 \pm 0.057\text{ }\mu\text{g/L}$
171 and $10.69 \pm 1.71\text{ }\mu\text{g/L}$; VLF = $0.087 \pm 0.034\text{ }\mu\text{g/L}$ and $7.48 \pm 2.25\text{ }\mu\text{g/L}$; CBZ = $0.079 \pm 0.018\text{ }\mu\text{g/L}$ and
172 $10.05 \pm 0.87\text{ }\mu\text{g/L}$. During the exposure period, mixture concentrations were re-established daily 1 h
173 after seawater renewal and mussel feeding to prevent any compounds adsorption on algae. No
174 mortality was recorded during the 7-day exposure period. Genetic determination of the species
175 confirmed the absence of hybridization between *Mytilus galloprovincialis* and *Mytilus edulis*
176 (Laboratory TOXEM, Montivilliers, France).

177 Mussels were collected for dissection at the end of the exposure period. The digestive glands
178 and the remaining soft tissues were frozen at -80°C in cryotubes prior to analysis. In order to avoid any
179 bias due to the sex confounding factors, as males and females can respond differently to contaminant
180 exposures (Dumas et al., 2020a; Ramirez et al., 2022), the sex of mussels was determined. Microscopic
181 analysis of a gonadal smear allowed us to identify the presence of spermatozoa or oocytes and thus
182 define the sex of the individuals. We identified 17 males and 18 females in SC exposure conditions; 16
183 males and 19 females in LD exposure conditions, and 22 males and 13 females in HD exposure

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Commenté [DT3R1]: J'ai rajouté tous les jours

184 conditions. Only male mussel samples were used for subsequent analyses because of the greater
185 number of males identified per condition with sufficient material to carry out both metabolomic and
186 proteogenomics analyses on the same sample. Twelve samples from each exposure condition were
187 prepared for omics analyses, thereby ensuring suitable statistical conditions.

188 2.3. Metabolomics analysis

189 2.3.1. Sample preparation

190 Frozen digestive glands of male mussels were freeze-dried using the Heto powerDry LL3000
191 (Thermo Fisher Scientific), and then ground into powder. As described in previous studies (Bonnefille
192 et al., 2018a; Dumas et al., 2022a; Ramirez et al., 2022), 30 mg of powder was homogenized in 240 μ L
193 of methanol and 75 μ L of ultrapure water was added and further vortexed for 60 sec. Then 240 μ L of
194 dichloromethane and 120 μ L of ultrapure water were added and vortexed for 60 sec. The samples
195 were left on ice for 15 min, vortexed, and centrifuged at 2,000 \times g for 15 min at 4°C. Then, 50 μ L of the
196 supernatant was transferred into glass tubes and evaporated to dryness under a nitrogen stream at
197 35°C with a TurboVap® LV concentration workstation from Caliper LifeSciences (Waltham, MA, USA).
198 The extracts were re-suspended in 200 μ L of water/acetonitrile (95/5; v/v), and then filtered into
199 centrifugal devices with a 10 kDa cut-off (VWR, Fontenay-sous-Bois, France). The filtrates were finally
200 transferred into vials prior to liquid chromatography–high resolution mass spectrometry (LC-HRMS)
201 analysis.

202 2.3.2. Data acquisition and quality control

203 LC-HRMS untargeted analysis was performed on a Thermo Scientific Vanquish™ Quaternary
204 HPLC system (Waltham, MA, USA) coupled with a Thermo Scientific Q Exactive™ Focus Hybrid
205 Quadrupole-Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI) probe.

206 HPLC separation was performed with a reverse phase pentafluorophenylpropyl (PFPP)
207 analytical column (100 \times 2.1 mm; 3 μ m particle size; Sigma Aldrich, PA, Bellefonte, USA). Each sample
208 (10 μ L) was loaded onto the column with full loop injection. Mobile phases were mixtures of

209 water/acetonitrile (99:1, v/v) (phase A) or water/acetonitrile (1:99, v/v) (phase B) spiked with 0.1% of
210 formic acid. The flow rate was set at 250 $\mu\text{L}/\text{min}$, and a gradient elution was started with 5% B until 3
211 min, increased until 40% B at 8 min, 50% B at 9 min, 70% B at 13 min, to reach 95% B at 15 min until
212 18 min, and then returned to starting conditions 5% B at 21 min, followed by a 7-min re-equilibration
213 period.

214 The Q-Exactive™ Focus HRMS was adjusted to a mass resolution of 35,000 (FWHM, m/z 200)
215 with a mass range of 50–750 m/z . Samples were analyzed simultaneously in positive and negative
216 electrospray ionization modes (ESI+ and ESI-, respectively) using polarity switching mode with the
217 following settings: spray voltage 3.35 |kV|, sheath gas flow rate 55, gas flow rate 10, S-Lens RF level
218 50, capillary temperature 300°C and heater temperature 250°C. The sample injection order was
219 randomly determined to reduce the influence of potential confounding factors. MS/MS acquisitions
220 were performed on QC samples with a high energy collision dissociation cell set at 20 eV with a mass
221 resolution of 17,500.

222 A quality control (QC) sample corresponding to a pool of 30 μL of each sample extract was
223 injected 10 times at the beginning of the analytical sequence to equilibrate the column. QC injections
224 were repeated throughout the sequence to monitor the analytical repeatability and sensitivity.
225 Relative standard deviations (RSD) were calculated for each ion detected in the QC samples to assess
226 the injection repeatability. An analytical variability control was set such that 70% of ions had to have
227 an RSD <30% (Want et al., 2010).

228 2.3.3. *Data pre-processing*

229 The raw data files were converted into mzXML files with MSConvert freeware (ProteoWizard
230 3.0) (Kessner et al., 2008). A multi-step strategy was applied for processing ESI+ and ESI- acquisitions
231 separately using the XCMS package (Smith et al., 2006) in the R environment. Optimized XCMS
232 parameters were implemented as follows: the m/z interval for peak picking was set at 0.0025, the
233 signal-to-noise ratio threshold was set at 10, the group bandwidth was set at 8, and the minimum

234 fraction was set at 0.5. XCMS returned results as a peak table with variable identity (i.e. m/z and
235 retention time) and feature abundances (i.e. peak area). After visual inspection of each extracted ion
236 chromatogram, all features corresponding to baseline drift or background noise were discarded from
237 the peak table. In addition, the CAMERA Bioconductor package (Kuhl et al., 2012) was used to remove
238 isotopes, adducts, and fragments from the peak table, thus avoiding information redundancy. Based
239 on QC sample injections, only features with an RSD < 30% were kept for further data processing and
240 annotation.

241 *2.3.4. Metabolite annotation and identification*

242 Metabolite annotation was carried out by mass-matching with 0.002 Da precision using the
243 Human Metabolome Database (HMDB; <http://www.hmdb.ca/>; (Wishart et al., 2013)). The annotation
244 confidence levels were defined according to the recommendations of the Compound Identification
245 work group of the Metabolomics Society (Blaženović et al., 2018): (i) level 1 corresponded to
246 unambiguous identifications based on the accurate mass and retention time of the corresponding
247 analytical standard injected under the same analytical conditions (in-house database based on more
248 than 300 metabolites belonging to amino acids, nucleotides, carbohydrates, lipids, cofactors and
249 vitamins), and (ii) level 2 corresponded to putative annotations based upon physicochemical
250 properties and/or spectral similarity of MS/MS spectra with public databases (HMDB and mzCloud).

251 *2.3.5. Differential metabolite abundance analysis*

252 Differential metabolite abundance between the solvent control conditions and the CBZ-DCF-
253 VLF mixture treated conditions (SC vs LD or SC vs HD) was assessed with a Welch t-test. The false
254 discovery rate (FDR) was controlled using the Benjamini–Hochberg FDR correction. Metabolites with
255 an absolute significant modulation > 30% and an adjusted p-value < 0.05 were considered statistically
256 relevant. Modulation trend can also be of interest (p-value < 0.1). The rationale to set this threshold
257 was driven by the potential to uncover a biological modulation trend resulting from the exposure.
258 Focusing on individual metabolites in isolation represents a somewhat simplified perspective of

259 metabolic changes that encompass intricate interconnections within metabolic pathways. In fact,
260 observing a trend ($p < 0.1$) indicating either a decrease or increase in multiple metabolites within the
261 same pathway, thereby highlighting a disturbance in the biochemical cascade, might hold more
262 biological significance than relying solely on a single marker metabolite ($p < 0.05$).

263 2.3.6. *Metabolic pathway analysis*

264 Pathway analysis was performed on MetaboAnalyst 5.0 (Xia and Wishart, 2010) in order to
265 highlight significantly impacted metabolic pathways. The Pathway analysis tool integrates both a
266 pathway enrichment analysis and a pathway topology analysis. The settings used are as follows:
267 hypergeometric test for enrichment method and relative-betweenness centrality for topology analysis.

268 2.4. Proteogenomics analysis

269 2.4.1. *Protein extraction*

270 Powder of each lyophilized digestive gland (7 mg) was mechanically homogenized by bead-
271 beating in 70 μ L of LDS sample buffer (Invitrogen, Fisher Scientific, Illkirch, France) with a Precellys
272 Evolution sample homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The
273 homogenates were centrifuged at 10,000 \times g for 3 min in order to pellet the cellular debris, and the
274 resulting supernatant was transferred to a new tube. As previously recommended (Hayoun et al.,
275 2019), samples were homogenized with a mix of 0.1 mm and 0.5 mm glass beads and 0.1 mm silica
276 beads. Five homogenization cycles at 6,800 rpm with a 30 s pause between each were necessary to
277 achieve complete dissolution of the material. Centrifugation at 16,000 \times g for 2 min was performed, and
278 samples were then incubated at 99°C for 5 min and briefly centrifuged. A 20 μ L aliquot of each
279 supernatant was then subjected to SDS-PAGE for a short electrophoretic migration, as described
280 previously (Trapp et al., 2015). The whole-protein content from each well was extracted as a single
281 polyacrylamide band, processed as described (Hartmann et al., 2014), and submitted to proteolysis
282 with trypsin (Roche) using 0.01% ProteaseMAX surfactant (Promega).

283 2.4.2. *Liquid chromatography - tandem mass spectrometry method*

284 Shotgun proteomics peptide analysis was carried out using a Q Exactive HF mass spectrometer
285 (ThermoFisher Scientific) coupled with an UltiMate 3000 LC system (Dionex-LC Packings). The
286 analytical method was previously described in Klein et al. (2016). Briefly, peptides were resolved onto
287 a nanoscale C18 PepMapTM 100 capillary column (LC Packings) at a 0.2 µl/min flow rate with a 90-min
288 gradient of acetonitrile and 0.1% formic acid. A Top20 data-dependent method was used, which
289 consisted of scan cycles involving a full scan of peptide ions in the Orbitrap analyzer, followed by high-
290 energy collisional dissociation and MS/MS scans on the 20 most abundant precursor ions. Full scan
291 mass spectra were acquired from *m/z* 350 to 1,800 at 60,000 resolution. Ion selection for MS/MS
292 fragmentation and measurement was performed by applying a 10 s dynamic exclusion window.

293 2.4.3. *Peptide assignment and proteomics data analysis*

294 MS/MS spectra were assigned to peptide sequences by the MASCOT Daemon 2.3.2 search
295 engine (Matrix Science, London, UK), with searches performed against the digestive gland specific
296 RNAseq-derived database obtained by *de novo* assembly with Trinity version 2.4 (Haas et al., 2013),
297 followed by ORF search and transcript translation with Transdecoder, as described in Cogne et al.
298 (2019). The final protein database contained 86,022 putative protein sequences totaling 29,583,007
299 amino acids, as reported in Dumas et al. (2022). For MS/MS spectra assignment, the parameters were:
300 full-trypsin specificity, maximum of one missed cleavage, mass tolerance of 5 ppm on the parent ion
301 and 0.02 Da on the MS/MS, carboxyamidomethylated cysteine (+57.0215) as a fixed modification, and
302 oxidized methionine (+15.9949) and deamidation of asparagine and glutamine (+0.9848) as variable
303 modifications. All peptide matches presenting a MASCOT ion score with a p-value of < 0.05,
304 corresponding to an FDR of 1% as evaluated with the DecoyPyrat procedure (Wright and Choudhary,
305 2016), were filtered and assigned to a protein based on a parsimony rule (with at least two different
306 peptides, one of which was unique). Functional annotation of identified proteins was performed by
307 sequence similarity search using Swissprot and NCBIInr databases with the DIAMOND tool alignment,

308 as previously described (Trapp et al., 2018). Peptide-to-spectrum matches were counted for each
309 protein leader of the protein group defined on the basis of their sequence similarities.

310 2.4.4. *Mass spectrometry and proteomics data*

311 The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium
312 via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD045957 and
313 project 10.6019/PXD045957.

314 2.4.5. *Differential protein abundance analysis*

315 For each validated protein, the number of spectral counts was used as a proxy of their relative
316 abundances and was normalized, as described in Liu et al. (2004). Differential protein abundance
317 between the solvent control condition and the CBZ-DCF-VLF mixture treated conditions (SC vs LD or SC
318 vs HD) was assessed with a Student *t*-test, followed by a Benjamini-Hochberg correction of the p-value.
319 Proteins with an absolute significant modulation > 40% and an adjusted p-value < 0.05 were considered
320 as differentially expressed between the solvent control condition and the treated conditions.

321 2.4.6. *Function and pathway enrichment analysis*

322 Gene Ontology (GO) term enrichment analysis was performed on the STRING database
323 (Szklarczyk et al., 2019; <https://string-db.org/>). Enrichment analysis of Kyoto Encyclopedia of Genes
324 and Genomes (KEGG) pathways was performed using a web-based tool, i.e. the KEGG Orthology-Based
325 Annotation System, intelligent version (KOBAS-i) (Bu et al., 2021). The *Mizuhopecten yessoensis* bivalve
326 mollusk was used as reference species for the KEGG pathway enrichment analysis. For both GO terms
327 and KEGG pathway enrichment analysis, an enrichment ratio was calculated as follows:
328 Enrich ratio = $\text{Log}_{10}(\text{observed}/\text{expected})$. This was the ratio between: i) the number of proteins in the
329 network annotated with a given term, and ii) the number of proteins expected to be annotated with
330 this term in a random network of the same size. Enrichment ratios > 1 with an adjusted p-value < 0.05
331 (FDR; Benjamini-Hochberg) were considered relevant.

332 2.5. Metabolomics and proteogenomics data fusion

333 A detailed description of this mid-level data fusion strategy was described in Dumas et al.
334 (2022a). Briefly, variables of the different blocks (i.e. dataset named MetaboESI+, MetaboESI- and
335 Proteo) were scaled to unit variance. MCUVE-PLS (Cai et al., 2008) and consensus OPLS-DA (Boccard
336 and Rutledge, 2013) models were computed with combinations of toolboxes and in-house functions
337 in the MATLAB® 8 environment (The MathWorks, Natick, USA). MCUVE-PLS was carried out on the
338 three blocks using the libPLS 1.98 package (Li et al., 2018) from a population of 10^4 models with a
339 calibration sample to total sample ratio of 0.7. A reliability index threshold of 2 was applied to remove
340 variables considered uninformative and then reduce the dimensionality of the three blocks. Consensus
341 OPLS-DA was then implemented using the K-OPLS package (Bylesjö et al., 2008) for fusion of the
342 remaining data subsets, while comparing the solvent control (SC) to each exposure condition
343 separately, i.e. low concentration 0.1 µg/L (SCvsLD) and high concentration 10 µg/L (SCvsHD). Both
344 models were found to be optimal with two components (one predictive and one orthogonal) using
345 leave-one-out cross-validation. The variable loadings provided by each consensus OPLS-DA model
346 were then displayed on a shared and unique structures (SUS) plot to differentiate variables with
347 common or specific patterns of variation between both exposure conditions. Thresholds were set to
348 select the extreme variables of the SUS-plot, i.e. considered to be the most informative to explain the
349 effects of the CBZ-DCF-VLF mixture at both concentrations on the protein and metabolite modulation.
350 This variable selection strategy is expected to highlight correlated signatures of metabolites and
351 proteins with potential biological significances.

352 3. Results

353 3.1. Performance of metabolomics, proteomics and data integration

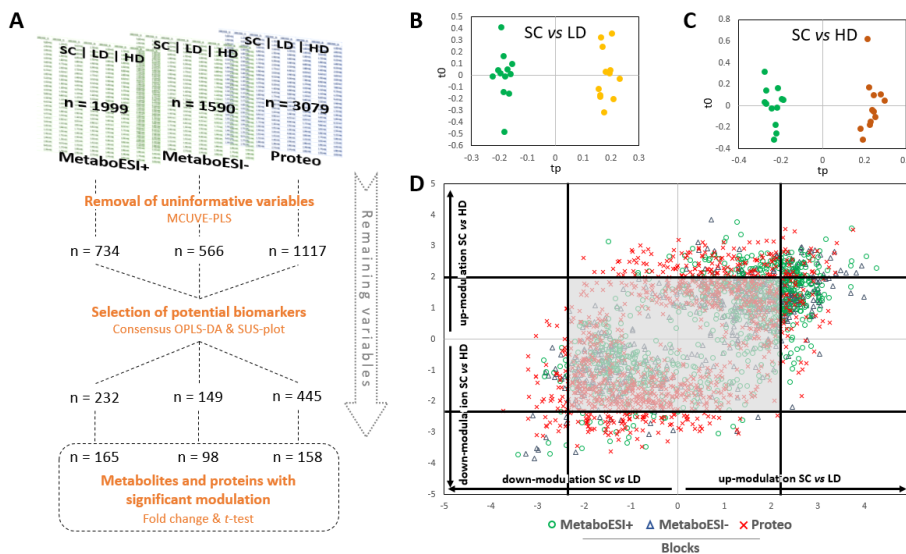
354 The metabolic fingerprints consisted of 2,660 features in ESI- and 3,821 features in ESI+. The
355 quality of the LC-HRMS acquisitions was validated as 77% of the features had a good analytical
356 repeatability in both ESI modes across QC replicates (RSD < 30%). The datasets were then filtered for

357 subsequent processing (removal of isotopes and features with an RSD < 30% or a retention time
 358 outside relevant MS analysis period, e.g. <60 s or >1,250 s), resulting in 1,590 features in ESI- and 1,999
 359 in ESI+.

360 The shotgun proteomics analysis generated 1,067,776 peptide spectra matches with a p-value
 361 of 0.05 or lower from the 2,061,124 recorded spectra (on average 52% of spectra interpreted). A total
 362 of 4,558 proteins were identified. Proteins that accumulated less than 30 spectral counts among all
 363 samples were filtered out, thereby resulting in a total of 3,079 proteins.

364 Regarding consensus OPLS-DA models, the SCvsLD model resulted in R2Y = 0.988 and Q2Y =
 365 0.826 (Figure 1B), while the SCvsHD model gave R2Y = 0.983 and Q2Y = 0.906 (Figure 1C), indicating
 366 that each model explained > 98% of the data variability and had a predictive performance of > 82%.

367 Hence, variables were selected on the SUS-plot and included subsets of 232, 149 and 445
 368 variables from the MetaboESI+, MetaboESI- and Proteo blocks, respectively (Figure 1A).

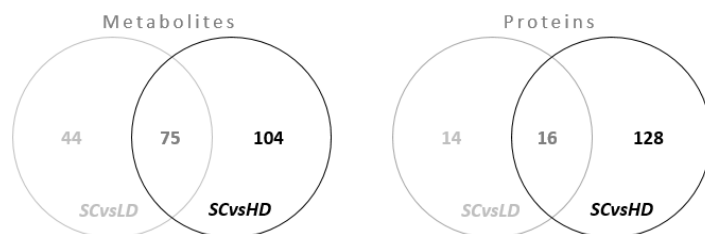


369
 370 **Figure 1:** Mid-level data fusion strategy based on MCUVE-PLS and consensus OPLS-DA analyses from the respective
 371 MetaboESI+, MetaboESI- and Proteo blocks. **(A)** The remaining variables from each block after each processing step. Score
 372 plots of the two consensus OPLS models with one orthogonal component (to) and one predictive component (tp): **(B)** solvent
 373 control exposure versus the low exposure concentration (SCvsLD) and **(C)** solvent control versus the high concentration
 374 (SCvsHD). **(D)** Shared and unique structures (SUS) plot integrating both SCvsLD and SCvsHD comparisons with common and
 375 specific modulations. Variables with a blue triangle belong to the MetaboESI+ block, those with a green circle to the

376 *MetaboESI- block and those with a red cross to the Proteo block. Thresholds are represented with black lines and less relevant*
377 *variables are shaded.*

378 The identification efforts were made on the variables selected by the consensus OPLS-DA
379 models. Among these selected variables, 30 metabolites were annotated from the MetaboESI+ block,
380 31 metabolites from the MetaboESI- block and 343 proteins from the Proteo block. The metabolite
381 and protein annotations are provided in Tables S1 and S2, respectively. Differential analysis of the
382 abundance of annotated metabolites and proteins for both SCvsLD and SCvsHD comparisons
383 (modulation amplitude and p-value) are given in Tables S1 and S2. Information on metabolites such as
384 metabolic pathway, RSD QC, mass error, retention time, etc., are also available in Table S1.

385 The Venn diagrams provided the number of metabolites or proteins significantly modulated in
386 a common or specific way depending on the exposure conditions (SCvsLD or SCvsHD) (Figure 2).
387 Overall, greater effects on the metabolome and proteome of male mussels were observed for the high
388 exposure concentration. Moreover, metabolites were more sensitive than proteins in revealing
389 molecular changes triggered by the low exposure concentration.



390
391 **Figure 2:** Venn diagrams highlighting specific and common modulations of metabolites or proteins when comparing solvent
392 control exposure versus low concentration exposure (SCvsLD), and solvent control versus high concentration (SCvsHD). Results
393 are given for the selected variables according to the SUS plot.

394

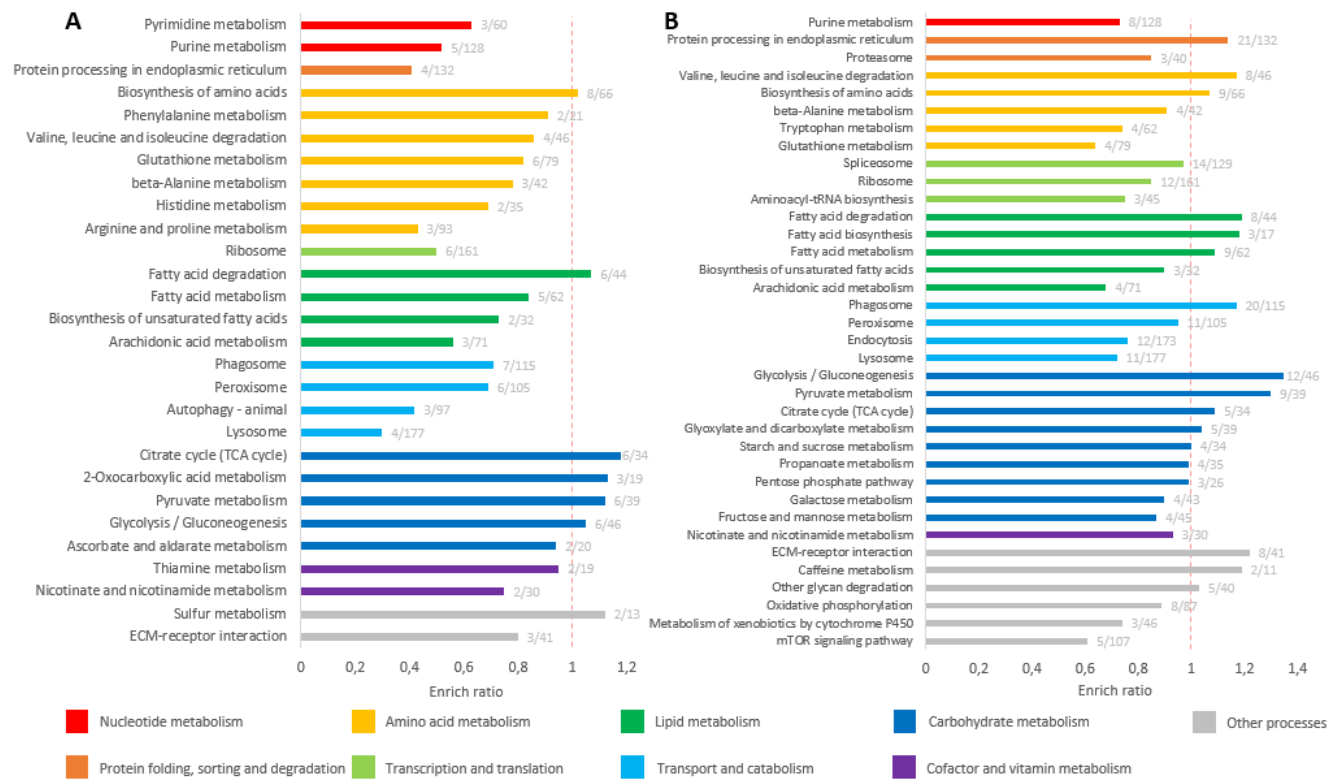
395 3.2. Mixture effects on proteome and metabolome

396 At the proteome level, the most significantly enriched KEGG pathways (Figure 3) were mainly
397 related to carbohydrate, lipid, nucleotide and amino acid metabolisms, as well as protein synthesis and
398 degradation processes. While only few of these pathways had an enrichment ratio of > 1 for the SCvsLD

399 condition (Figure 3A; n=7), more significantly enriched pathways were noted for the SCvsHD condition
400 (Figure 3B; n=14). Regarding GO term enrichment analysis, the results for both exposure conditions
401 (SCvsLD and SCvsHD) were categorized according to the biological process (Figure S1), molecular
402 function (Figure S2) and cellular component (Figure S3). Overall, these results corroborated those of
403 the KEGG pathway enrichment analysis and are presented in the Supplementary Data.

404 At the metabolome level, the modulated metabolites were mainly related to carbohydrate,
405 lipid, nucleotide and amino acid metabolisms, as well as cofactors and vitamins (Figure 4). Pathway
406 analysis revealed two significantly enriched metabolic pathways at both exposure concentrations,
407 including purine, nicotinate and nicotinamide metabolisms (Figure S4).

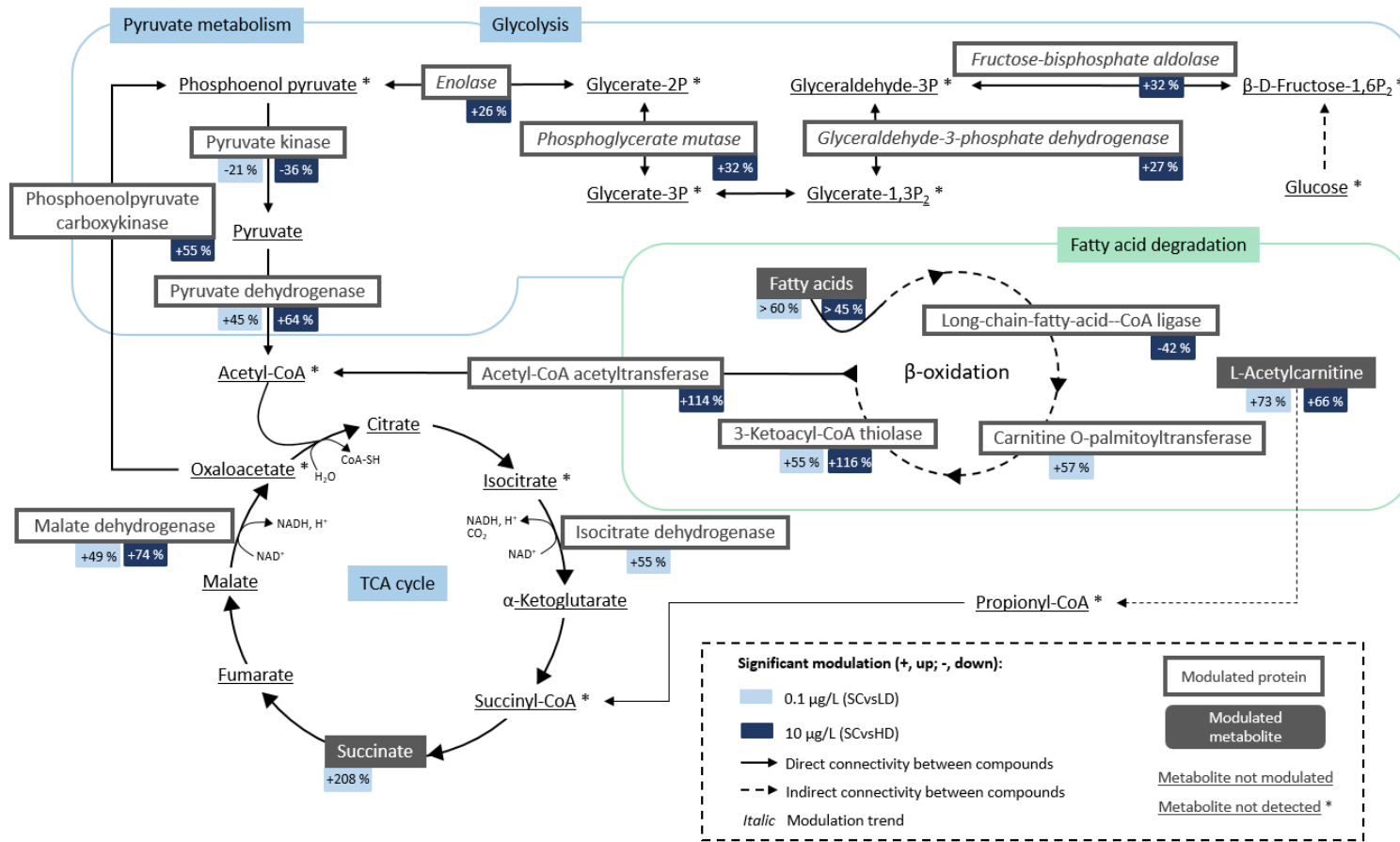
408 When considering both proteome and metabolome levels, consistent changes in protein and
409 metabolite abundances were observed, as outlined in the following sub-sections.



410

411 **Figure 3:** KEGG pathway enrichment analysis with proteins selected via consensus OPLS-DA models, while considering (A) the low exposure concentration (0.1 µg/L; SCvsLD) or (B) the high
 412 exposure concentration (10 µg/L; SCvsHD). The label on each bar corresponds to the number of selected proteins associated with the pathway relative to the total number of proteins in that
 413 pathway.

429 dehydrogenase [NAD] subunit gamma enzymes (Figure 5, Table S2), as well as the succinate metabolite
430 (Figures 4 and 5).



431

432 **Figure 5:** Metabolic pathways of carbohydrate and lipid metabolism mapping proteins and metabolites modulated at low (SCvsLD) and high (SCvsHD) mixture exposure concentration.

433 3.2.2. *Lipid metabolism*

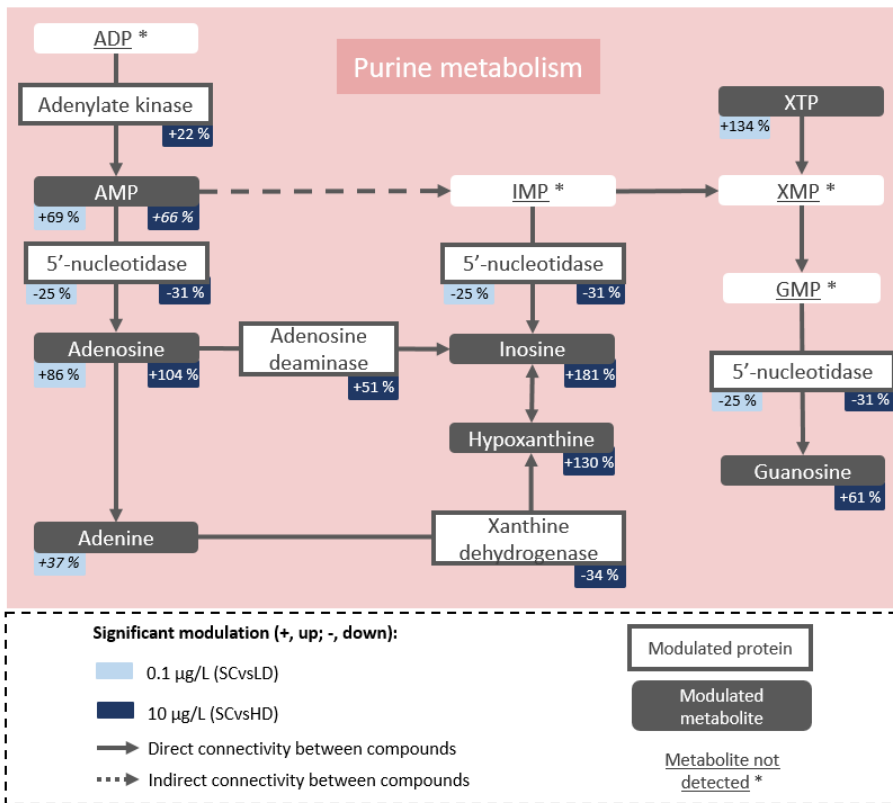
434 Among the lipid metabolism, fatty acid degradation through β -oxidation in mitochondria was
435 the main enriched pathway (Figure 3). Fatty acid degradation was also involved in energy production
436 by providing acetyl-CoA to the TCA cycle (Figure 5). Modulation of three enzymes responsible for β -
437 oxidation of fatty acids was observed (Figure 5, Table S2), along with up-modulation of acetyl-CoA
438 acetyltransferase 1, which catalyzed the last step in the transformation of fatty acids into acetyl-CoA.
439 Note that acetyl-CoA acetyltransferase was involved in other metabolic pathways, particularly in
440 pyruvate metabolism and amino acid metabolism e.g., valine, leucine and isoleucine degradation,
441 lysine degradation, as well as tryptophan metabolism.

442 Several metabolites associated with fatty acid metabolism were up-modulated at both
443 exposure concentrations. Among them, four acylcarnitine compounds, including L-acetylcarnitine,
444 were involved in energy production in mitochondria through beta-oxidation of fatty acids. In addition,
445 three acylglycine compounds considered to be fatty acid oxidation metabolites were up-modulated
446 (Figure 4, Table S1). L-acetylcarnitine plays an important role in the metabolism of both carbohydrates
447 and lipids, leading to an increase in ATP generation. L-acetylcarnitine is transported to the
448 mitochondria where it is transformed into free carnitine (detected but not modulated) and propionyl-
449 CoA (not detected). The latter is converted into succinyl-CoA (not detected) and finally to succinic acid,
450 which is involved in the TCA cycle. Consequently, the TCA cycle activity was enhanced, as highlighted
451 by the high modulation of succinic acid and two other enzymes (Figures 4 and 5).

452 3.2.3. *Nucleotide metabolism*

453 Among nucleotide metabolism, purine metabolism was highly affected, with four up-
454 modulated metabolites noted at the SCvsLD concentration and five up-modulated metabolites at the
455 SCvsHD concentration (Figure 6, Table S1). Modulation of these metabolites was in accordance with
456 proteomics results. Indeed, three proteins catalyzing metabolic reactions of these metabolites were

457 modulated in addition to adenosine deaminase (SCvsHD: +51%; $p < 0.01$; not selected by the models)
 458 (Figure 6).



459 **Figure 6:** Metabolic pathway of purine metabolism represented by consistent modulations of metabolite and protein
 460 abundance in the digestive gland of *Mytilus galloprovincialis* male mussels exposed at the low (SCvsLD; 0.1 µg/L) or high
 461 (SCvsHD; 10 µg/L) exposure concentration. ADP: adenosine 5'-diphosphate; AMP: adenosine monophosphate; IMP:
 462 inosine monophosphate; XMP: xanthosine 5'-phosphate; XTP: xanthosine 5'-triphosphate.

463 3.2.4. Amino acid metabolism

464 Amino acid metabolism was mainly characterized by the disturbance of four metabolites from
 465 cysteine and methionine metabolism (Figure 4, Table S1). Down-modulation of methionine sulfoxide
 466 was consistent with the up-modulation of peptide methionine sulfoxide reductase MsrA (SCvsHD:
 467 +75%; $p < 0.01$) which catalyzes the reduction of protein-bound methionine sulfoxide back to
 468 methionine, although MsrA was not selected by the models.

469 Glutathione metabolism had an enrichment ratio < 1 (Figure 3), yet up-modulation of
470 glutathione peroxidase catalyzing the oxidation of glutathione (reduced state; GSH) into glutathione
471 disulfide (oxidized glutathione; GSSG) was measured at the two exposure concentrations (Table S2).
472 GSSG was up-modulated, in agreement with the proteomic results (Figure 4, Table S1). While down-
473 modulation of GSH would be expected in this case, its up-modulation could indicate GSH *de novo*
474 synthesis. However, caution is needed in interpreting this result as GSH was annotated at level 2.
475 Modulation of GSSG and GSH could also be related to up-modulation of glutathione S-transferase
476 sigma 1 (Table S2), which allows GSH conjugation to endogenous substrates or xenobiotics.

477 3.2.5. *Cofactors and vitamin metabolism*

478 Regarding vitamin metabolism, nicotinate and nicotinamide metabolism was the most
479 enriched pathway (Figure S4). Nicotinic acid, i.e. known as vitamin B3, was up-modulated at the high
480 exposure concentration, while niacinamide was up-modulated to the same extent at both exposure
481 concentrations (Figure 4, Table S1). Nicotinic acid and niacinamide are precursors of the coenzymes
482 nicotinamide-adenine dinucleotide (NAD⁺) and nicotinamide-adenine dinucleotide phosphate
483 (NADP⁺). These coenzymes are involved in a myriad of metabolic processes, including energy
484 metabolism, mitochondrial functions, calcium homeostasis, antioxidation/generation of oxidative
485 stress, gene expression, immunological functions and cell death.

486 3.2.6. *Protein synthesis and degradation processes*

487 Upstream of protein synthesis, modulations were first observed at the transcription
488 (spliceosome) and translation (ribosome, aminoacyl-tRNA biosynthesis) levels, mainly under the
489 SCvsHD condition (Figure 3, Table S2). From spliceosome, splicing factor, arginine/serine-rich 4 was up-
490 modulated while splicing factor 3B subunit 3 was down-modulated (Table S2). Ribosomal proteins such
491 as 40S ribosomal protein S6 was up-modulated and four other ribosomal proteins were modulated to
492 a lesser extent. In addition, four proteins involved in aminoacyl-tRNA biosynthesis were up-modulated,
493 including glycine-tRNA ligase and cysteine-tRNA ligase (Table S2), as well as tryptophan-tRNA ligase

494 (+43%; $p < 0.05$) and leucine-tRNA ligase (+91%; $p < 0.005$) which were not selected by the models.

495 The cellular machinery is thus remodeled for specific protein synthesis in response to stress.

496 A high number of proteins ($n = 21$) selected by the SCvsHD model were related to protein
497 processing in the endoplasmic reticulum (ER). These modulated proteins are involved in the folding
498 and sorting of newly synthesized proteins. Among them, significant modulation of the ERGIC-53
499 protein, ribosome-binding protein 1 and B-cell receptor-associated protein 31 was observed, while
500 lower modulation was noted for DnaJ homolog subfamily A member 1, endoplasmic reticulum
501 chaperone BiP, HSP 70.3, protein disulfide isomerase and protein disulfide isomerase family A member
502 6 (Table S2). Accumulation of misfolded and unfolded proteins can induce ER stress. To re-establish
503 the ER function and restore homeostasis, degradation pathways are activated, such as the ER-
504 associated protein degradation (ERAD) pathway or the unfolded protein response (UPR) signalling
505 pathway. In this study, significant up-modulation occurred for RuvB-like 2 from the ERAD pathway, and
506 DnaJ homolog subfamily C member 3 from the UPR signalling pathway (Table S2). Hence, protein
507 synthesis seemed to increase in response to the high exposure concentration, as well as ER stress
508 induced by the accumulation and degradation of unfolded and misfolded proteins.

509 3.3. Targeting the molecular signatures of CBZ, DCF and VLF

510 In this section we discuss the nature of the molecular effects, while questioning whether they
511 resulted from interactive or additive effects. We hypothesized that if there is no interactive effect then
512 we should observe an addition of molecular signatures similar to those reported under individual
513 exposure conditions in previous studies. Inversely, interactions between the PhACs should lead to
514 molecular signatures different from those obtained previously with individual exposure.

515 In order to target specific molecular signatures, we reviewed the literature on molecular
516 effects triggered by single exposure of aquatic organisms to CBZ, DCF or VLF involving omics
517 approaches (metabolomics, proteomics and transcriptomics). We found 15 articles, 5 of which were
518 related to CBZ, 6 to DCF and 4 to VLF, including our previous publications focused on these three PhACs
519 individually. Among these studies, 6 were conducted on molluscs, 6 on fish, 2 on crustacean and 1 on
520 ascidian. The effects reported are described in Table S3, including modulated metabolites, proteins or
521 transcripts, the most enriched metabolic pathways, GO terms and KO. Based on these reviewed data
522 and the results obtained in this study, we have proposed an overview of common molecular effects as
523 well as common enriched GO terms and KO (Table 1). Hence, the reported proteins, metabolites, GO
524 terms and KO in Table 1 could correspond to the signature of CBZ, DCF or VLF within the observed
525 mixture effects. Proteins, metabolites, GO terms and KO that were only observed for one of the
526 pharmaceuticals, as highlighted in bold in Table 1 could therefore be considered as a specific signature
527 of this PhAC in the mixture. For instance, the molecular signature of CBZ consisted of 21 proteins and
528 1 metabolite that had been modulated in previous studies with CBZ alone and with the mixture in the
529 present study. Among these 21 proteins, 16 proteins (in bold) were only observed for CBZ (not
530 modulated in previous studies for DCF and VLF exposures) and could thus be considered as a specific
531 signature of CBZ effects in the mixture. In terms of GO and KO, the ER unfolded protein response,
532 protein synthesis and degradation, and binding processes seemed to be specific to the CBZ signature.
533 The molecular signature of DCF had the highest number of metabolites ($n = 7$) modulated by exposure

534 to the mixture. However, no specific signature of GO terms and KO was assigned to DCF. Regarding the
535 VLF signature, only a few proteins and metabolites (3 and 2, respectively) were specific, while changes
536 in glutathione metabolism and valine, leucine and isoleucine degradation seemed to be a specific
537 response of VLF in the mixture.

538 Hence, a moderate part of the observed modulations (32 proteins and 10 metabolites)
539 corresponded to the known molecular signatures of these three PhACs (Table 1) and may have resulted
540 from an additive effect. Furthermore, a major part of the modulated proteins and metabolites (Table
541 S1 and S2) had not been reported in previous exposures to PhAC alone. However, these proteins and
542 metabolites were mostly involved in the KO and GO terms already modulated by individual exposure
543 (Table 1). The response to these three PhACs will be discussed in the following section, while taking
544 their known MeOAs into account.

545
546

Table 1: Signature of carbamazepine, diclofenac and venlafaxine observed in both single exposure conditions from the literature and under the current mixture exposure conditions. Bold annotations correspond to proteins/transcripts, metabolites or GO terms and KO specific to one of the three pharmaceuticals. Metabolites that tended to be modulated are in italics

Contaminant	Signature of proteins or transcripts	Signature of metabolites	Signature of GO terms and KO	References
Carbamazepine (CBZ)	40S ribosomal protein S6; ATP synthase subunit beta, mitochondrial; Calmodulin; Cartilage matrix protein; Cathepsin L; Cathepsin L1; Carboxypeptidase B; Coatomer subunit beta'; Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B; Glutathione S-transferase sigma 1; Glycogen debranching enzyme; Histone H2A; Inositol-1-monophosphatase; Lachesin; Myophilin; Myosin VIIA; Protein ERGIC-53; RuvB-like 2; Thioredoxin-2; TNF ligand-like 1; Tubulin alpha chain	Adenosine	Acyl carnitine compounds; Cysteine and methionine metabolism; Endoplasmic reticulum unfolded protein response; Fatty acid oxidation; Metal ion binding; Nucleic acid binding; Phenylalanine metabolism; Protein folding; Protein synthesis and degradation; Purine metabolism; TCA cycle	Baratange et al 2022; Dumas et al 2022; Navon et al 2021; Yan et al 2018; Kovacevic et al 2016
Diclofenac (DCF)	ATP synthase subunits region ORF 7-like; Calmodulin; Cartilage matrix protein; Cathepsin L; Cathepsin L1; Cysteine--tRNA ligase, cytoplasmic; CD109; Dual oxidase 2; Glyceraldehyde-3-phosphate dehydrogenase; Malate dehydrogenase; Tryptophan--tRNA ligase, cytoplasmic; Tubulin alpha-1A chain	Acetylcarnitine; Adenine; Hypoxanthine; Inosine; Niacinamide; Nicotinic acid; Uridine	Acyl carnitine compounds; β -oxidation of very long chain fatty acids; Folding protein; Glucose metabolism; Nicotinate and nicotinamide metabolism; Pentose phosphate pathway; Phenylalanine metabolism; Purine metabolism; Pyrimidine metabolism; TCA cycle; Tryptophan metabolism; Tyrosine metabolism	Bonnefille et al 2018; Bouly et al 2022; Fu et al 2021; Schmidt et al 2013
Venlafaxine (VLF)	Histidine ammonia-lyase; Phosphoenolpyruvate carboxykinase, cytosolic [GTP]; Serine--pyruvate aminotransferase	Adenosine; Deoxyribose; N-Acetylvaline	Cysteine and methionine metabolism; Glucose metabolism; Glutathione metabolism; Lipid metabolism; Nicotinate and nicotinamide metabolism; Pentose phosphate pathway; Phenylalanine metabolism; Purine metabolism; Pyrimidine metabolism; Tryptophan metabolism; Tyrosine metabolism; Valine, leucine and isoleucine degradation	Hong et al 2022; Ramirez et al 2022; Costa et al 2021; Best et al 2014

547

548 4. Discussion

549 A combined metabolomics and proteogenomics approach revealed molecular changes in digestive
550 gland of male mussels (*Mytilus galloprovincialis*) exposed for 7 days to the CBZ-DCF-VLF mixture at low
551 and high concentration. Consistent observed proteome and metabolome changes were the result of
552 combined omics analyses carried out on the same samples (digestive glands), as well as of a mid-level
553 data fusion strategy, that led to the selection of correlated protein and metabolite signatures.
554 According to current knowledge, some of these statistically correlated signatures appear to be
555 biologically relevant. As discussed below, those changes are mainly related to energy metabolism,
556 protein synthesis, ER stress, defense mechanisms and oxidative stress. The known MeOAs of CBZ (e.g.
557 cAMP depletion, GABA receptor agonist, voltage-dependent Na⁺ and K⁺ channels interaction,
558 autophagy induction), DCF (e.g. cyclooxygenases inhibition and prostaglandins depletion) and VLF (e.g.
559 serotonin increase) were taken into consideration to gain insight into the mixture effects.

560 4.1. Energy metabolism and fatty acid oxidation

561 Overall, exposure to the CBZ-DCF-VLF mixture triggered a major energy metabolism response. The
562 TCA cycle, glycolysis, pyruvate metabolism and fatty acid degradation are interconnected pathways
563 whose ultimate goal is primarily to produce energy such as ATP. Such results are not surprising as the
564 digestive gland of bivalves plays an important role in the storage of metabolic reserves, thereby
565 providing energy during stress periods (Gosling, 2015). The increased energy demand caused by
566 contaminant exposure is a common metabolic response to detoxify the organism, maintain cellular
567 homeostasis and repair damage. For instance, depletion of energy reserves (lipid, glycogen and
568 protein) has been measured in digestive glands of *M. galloprovincialis* following 11 weeks exposure to
569 1.2 µg/L of the hypolipidemic drug atorvastatin (Falfushynska et al., 2019). Regarding the
570 pharmaceutical mixture tested in the present study, single exposure to CBZ, DCF or VLF have already
571 been found to disturb energy metabolism in aquatic organisms. In our previous study, exposure of
572 male mussels (*M. galloprovincialis*) to CBZ (0.08 and 8 µg/L; 3 days) led to the disruption of fatty acid

573 degradation in mitochondria and peroxisomes through the modulation of several enzymes involved in
574 fatty acid β -oxidation, as well as many fatty acids, acylcarnitines and acylglycines (Dumas et al., 2022a).
575 In the same way, DCF was also shown to alter fatty acid β -oxidation in various aquatic organisms (Bouly
576 et al., 2021; Fu et al., 2021). Fu et al. (2021) observed a modulation of several acylcarnitines in the
577 *Hyalella azteca* crustacean exposed to DCF (10 and 100 $\mu\text{g/L}$) for 10 days, revealing a perturbation in
578 the carnitine shuttle. The carnitine shuttle is a critical process for the transport of long-chain fatty acids
579 from cell plasma across the membrane to mitochondria subsequent to β -oxidation. Furthermore, L-
580 carnitine and L-acetylcarnitine were modulated in the *Lymnaea stagnalis* gastropod exposed for 16
581 weeks post-hatch to 0.1 $\mu\text{g/L}$ of DCF (Bouly et al., 2021). While VLF does not disrupt fatty acid β -
582 oxidation, it was reported to induce changes in transcript expression related to lipid metabolism,
583 glucose metabolism and energy metabolism measured in the marine fish *Dicentrarchus labrax*
584 following 21 days exposure at 0.01 and 1 $\mu\text{g/L}$ concentration (Costa et al., 2021). The combined effects
585 of these three PhACs on energy metabolism were thus conserved, although such effects could be
586 considered as a general response to cope with stress, and were not necessarily related to their
587 expected MeOAs.

588 4.2. Protein synthesis and endoplasmic reticulum stress

589 Exposure to the CBZ-DCF-VLF mixture also triggered an intensification of processes related to
590 protein synthesis, with subsequent ER stress induction, particularly at high concentration. Indeed,
591 numerous modulated proteins are involved in transcription (spliceosome), translation (ribosome,
592 aminoacyl-tRNA biosynthesis) and protein processing in ER (protein folding, sorting and degradation).
593 When the demand for protein folding increases (e.g. enhanced protein synthesis, accumulation of
594 mutated or abnormal proteins, etc.) and exceeds the protein folding capacity, misfolded/unfolded
595 proteins accumulate in the ER lumen and trigger ER stress (Foufelle and Fromenty, 2016). Such stress
596 has also been confirmed by activation of the unfolded protein response (UPR) through up-modulation
597 of the DnaJ homolog subfamily C member 3. When CBZ exposure was found to cause ER stress in *M.*

598 *galloprovincialis* digestive glands (Dumas et al., 2022a), we argued that CBZ induced autophagy
599 through ER stress induction (e.g. accumulation of misfolded proteins, changes in calcium [Ca²⁺] levels)
600 or inhibition of inositol monophosphatase, leading to free inositol depletion and reduced myo-inositol-
601 1,4,5-triphosphate. In this study, we observed both ER stress and down-modulation of inositol-1-
602 monophosphatase, although no proteins involved in autophagy were detected. These effects could
603 hence be related to the MeOA of CBZ. To our knowledge, ER stress induction caused by DCF or VLF in
604 aquatic organisms has not been previously reported. Otherwise, prolonged ER stress can affect the
605 organism's functions (protein synthesis and folding, lipid synthesis and Ca²⁺ homeostasis) and
606 definitely lead to cell death or apoptosis (Schwarz and Blower, 2016).

607 4.3. Defense mechanisms and oxidative stress

608 The CBZ-DCF-VLF mixture also triggered defense mechanisms at both exposure
609 concentrations, including the biotransformation process and protection against oxidative stress.
610 Glutathione S-transferases (GSTs), which were up-modulated in this study, are a family of phase II
611 biotransformation enzymes, catalyzing conjugation of the reduced form of glutathione (GSH) to
612 xenobiotics. Some GST isoforms are also capable of neutralizing lipoperoxidation products through
613 GSH oxidation to GSSG (Regoli and Giuliani, 2014). *M. galloprovincialis* exposure to CBZ (0.1 µg/L; 7
614 days) and DCF (1 and 1000 µg/L; 96 h) have already been shown to increase GST activity in digestive
615 glands (Martin-Diaz et al., 2009; Schmidt et al., 2011), while there is no such data on VLF exposure in
616 molluscs. An increase in GST activity is nevertheless a common response of *M. galloprovincialis* during
617 PhACs exposure as shown in previous studies (Afsa et al., 2023; Capolupo et al., 2016; Freitas et al.,
618 2019). GSTs could also be involved in the formation of thiol metabolites resulting from the oxidation
619 of CBZ, whereby GSH would be conjugated to the carbonyl group of CBZ (Vernouillet et al., 2010).

620 Metabolism enhancement (e.g. protein synthesis, fatty acid degradation, energy metabolism,
621 etc.) induced an increase in aerobic reactions, leading to the accumulation of reactive oxygen species
622 (ROS), which can be toxic to cells. Regarding the antioxidant defense system, glutathione peroxidase

623 (GPx) plays major role by catalyzing the reduction of hydrogen peroxide, organic hydroperoxide and
624 lipid peroxides using GSH (Bhagat et al., 2016). In this study, up-modulation of GPx, as well as GSSG,
625 revealed oxidative stress induction at both exposure concentrations. Further evidence of oxidative
626 stress was highlighted in this study, as for instance the up-modulation of mitochondrial peroxiredoxin-
627 5. This thioredoxin peroxidase is an antioxidant enzyme known to have high affinity for organic
628 peroxides and peroxynitrite, and protect mitochondrial DNA from oxidative attack (Banmeyer et al.,
629 2005; De Simoni et al., 2013). ROS and reactive nitrogen intermediates can cause damage to many
630 cellular components such as proteins. In the latter, methionine is one of the most easily oxidized amino
631 acids, resulting in its conversion to methionine sulfoxide. As a repair mechanism, peptide methionine
632 sulfoxide reductase (MsrA) catalyzes the reduction of methionine sulfoxide, both free and protein-
633 bound, back to methionine, the lack of which would cause severe cellular damage (Weissbach et al.,
634 2002). This repair mechanism occurred in the digestive gland of male mussels exposed to the CBZ-DCF-
635 VLF mixture, as MsrA was up-modulated at high concentration, consistent with the down-modulation
636 of methionine sulfoxide. The fact that oxidative stress occurred in this experiment was not surprising
637 in the light of previous findings. Oxidative stress is the most common effect of CBZ reported in bivalves
638 (Almeida et al., 2020). Induction of oxidative stress has also been frequently reported for DCF, although
639 this process was more time-, concentration- and organism-dependent (Bonnefille et al., 2018a). In
640 vertebrates, VLF has a protective effect against oxidative stress due to its dual activity on the reuptake
641 inhibition of both serotonin and norepinephrine (Eren et al., 2007). This mechanism has been
642 confirmed in oyster immunocytes in which norepinephrine down-regulates interleukins, which are key
643 elements required for ROS production (Lacoste et al., 2001). Hence, the oxidative stress observed in
644 this study could mainly have been induced by CBZ and DCF, whereas to our knowledge VLF does not
645 induce oxidative stress in aquatic organisms.

646 The molecular response of mussels was also highly marked by changes in purine metabolism.
647 Nucleobases (e.g. adenine, hypoxanthine) are the building blocks for the synthesis of nucleosides (e.g.

648 adenosine, inosine, guanosine and uridine), which were up-modulated in this study. For instance,
649 adenosine is well recognized as being part of ATP and ADP, which are crucial molecules in energy
650 production. The addition of ribose and phosphate groups to the nucleobases generates nucleotides
651 (e.g. AMP), which can be used in RNA synthesis, while the addition of deoxyribose (up-modulated in
652 this study) leads to precursors which are used by the organism *in vivo* to synthesize DNA. Hence, the
653 CBZ-DCF-VLF mixture could modify the turnover and repair mechanisms of genetic material. This
654 hypothesis is also supported by the observed up-modulation of histone H2A (Table S2) leading to
655 chromatin formation. Histones are subject to post-translational modifications which are intimately
656 related to replication, recombination and DNA repair (Bergink et al., 2006). Hence, DNA damage
657 through oxidative stress cannot be excluded.

658 Finally, the up-modulation of nicotinic acid and niacinamide (precursors of NAD⁺ and NADP⁺)
659 was consistent with the proteomic and metabolomic results described in the discussion. NAD⁺ and
660 NADP⁺ coenzymes are common fundamental mediators of various biological processes discussed in
661 this study, including energy metabolism, mitochondrial functions, calcium homeostasis,
662 antioxidant/oxidative stress induction and cell death (Ying, 2008). Interestingly, nicotinic acid and
663 niacinamide modulation has been observed in *L. stagnalis* exposed to DCF (Bouly et al., 2021) and
664 seems to be specific to the DCF molecular signature (Table 1). However, this modulation was not
665 related to the known DCF MeOA, which is based on COX inhibition.

666 4.4. Contribution of omics to the study of mixture effects

667 By selecting a mixture of PhACs with different therapeutic classes that do not share known
668 MeOAs, we initially hypothesized that if these PhACs do not interact in terms of molecular targets,
669 then the resulting molecular signatures would be the sum of the already reported individual signatures
670 of each PhAC. A possible limitation of this study to address such a question could be the lack of existing
671 knowledge about molecular effects of these PhACs in bivalves. In addition, mixture effects are not
672 strictly compared against individual exposures within the same study. Hence, comparison with

673 previous studies should be treated with caution due to differences in exposure conditions. Despite
674 these limitations, our study produces encouraging results for the continued use of multi-omics
675 approaches to investigate mixture effects.

676 Through our multi-omics approach, we observed similar effects of the CBZ-DCF-VLX mixture
677 compared to the effects induced by single exposure. Indeed, the molecular effects triggered by the
678 mixture, such as changes in energy metabolism and lipid metabolism, as well as oxidative stress
679 induction, increased protein synthesis and degradation followed by ER stress, have also been
680 previously reported for some or all of these three PhACs. Nevertheless, while certain effects of the
681 mixture could be linked to known individual effects of the PhACs, we did not detect any modulation
682 associated with the MeOA of DCF (e.g. cyclooxygenases and prostaglandins) or VLF (e.g. serotonin),
683 except for CBZ. This, however, should be carefully considered as many factors could explain why
684 modulations related to the MeOA were not observed (e.g. differences in exposure conditions, choice
685 of organisms and the absence of standardized methodology for omics). As described in the discussion,
686 ER stress induction and down-modulation of inositol monophosphatase could both be related to the
687 CBZ MeOA involved in autophagy induction. The stronger expression of the CBZ MeOA compared to
688 that of DCF and VLF may be related to the way CBZ is metabolized. Indeed, it is known that in human
689 CBZ can auto-induce its metabolization through the activation of hepatic cytochrome P450 (CYP)
690 enzymes (Ayano, 2016). Some CBZ metabolites such as carbamazepine-10,11-epoxide can also be
691 toxic, even more than CBZ (Heye et al., 2016). In addition, CYP induction by CBZ may also increase the
692 rate of metabolism of DCF or VLF. Indeed, CBZ can induce CYP3A4, CYP2C8 and CYP2C9 (Darwish et al.,
693 2015; Liu et al., 2015), which are also involved in DCF (Krasniqi et al., 2016) and VLF (Fogelman et al.,
694 1999) metabolization. Although CYP3A4 and CYP2C8 were detected in this study (results not shown),
695 no modulation was observed. Further research on the toxicokinetic interactions between these PhACs
696 is required to be able to further interpret these results.

697 Beyond potential toxicokinetic interactions, the overall molecular effects observed tended to
698 support our initial hypothesis, suggesting that the mixture effects were predominantly additive. Our
699 findings indicated that the multi-omics approach effectively identified the toxicity pathways associated
700 with each individual molecule in the mixture, thereby showcasing the advantage of this molecular-
701 based approach over apical-based endpoints for studying mixture effects. This advantage is particularly
702 beneficial when the effects are additive, as the interpretation can be based on existing knowledge,
703 which facilitates the detection of known effects or MeOAs. However, identifying interactive effects
704 (e.g. synergistic or antagonistic) through omics approaches may be more challenging due to the
705 complexity of the resulting molecular effects. To address this shortcoming, exposure to a mixture of
706 PhACs belonging to the same therapeutic class and sharing similar MeOAs could potentially serve as
707 training data for omics approaches to achieve more clear-cut detection of interactive effects. This
708 could be further enhanced by exploring exposure to a mixture of PhACs with interactions known to
709 trigger toxicity in humans. Such studies would also be very relevant to acknowledge effects on
710 situations likely to occur in the environment.

711 Our findings also revealed that omics approaches are effective in detecting mixture effects,
712 even at low concentrations. In fact, many of the effects observed at high concentration were also
713 detected at the low exposure concentration. This highlights the relevance of omics in addressing
714 environmental concerns as PhACs are commonly found at low concentration in the environment. Note
715 also that the ecotoxicity of a mixture of PhACs could exceed the effects of individual compounds, even
716 if each PhAC is present at a low concentration that would not individually cause significant toxicity to
717 exposed organisms (Backhaus, 2014). In addition to binary or ternary mixtures, it is crucial to consider
718 the realistic diversity of contaminants that occur in the environment in order to be able to fully
719 understand the impact of anthropogenic discharges. Omics approaches have been applied in studies
720 investigating the effects of environmental mixtures, such as wastewater treatment plant effluent, on
721 aquatic organisms (Bebiano et al., 2016; Berlioz-Barbier et al., 2018; Dumas et al., 2020b, 2020a;

722 Simmons et al., 2017). These studies integrated the complexity of real-world mixture scenarios,
723 thereby providing a more comprehensive understanding of the potential mixture effects of
724 contaminants in the environment.

725 5. Conclusion

726 Integration of metabolomics and proteogenomics approaches has proven to be very effective to
727 highlight consistent changes in metabolome and proteome of male mussels *Mytilus galloprovincialis*,
728 exposed 7 days to the CBZ-DCF-VLF mixture at a low and high concentration (0.1 and 10 µg/L). Such
729 consistent changes observed between the two molecular levels are the result of combined omics
730 analyses carried out on the same samples (digestive glands), as well as of a mid-level data fusion
731 strategy allowing the selection of correlated protein and metabolite signatures. Some of these
732 statistically correlated signatures have been shown to be biologically relevant, according to the current
733 knowledge. Such mixture-induced modulations of proteins and metabolites indicate a metabolic
734 disturbance, as well as endoplasmic reticulum stress and oxidative stress, even at the environmental
735 concentration, which can be damaging to marine bivalves under chronic exposure. The consideration
736 of the known MeOAs and molecular signatures of individual PhACs was essential to decipher the
737 mixture effects, thus revealing a potential additive effect. Finally, the present study clearly revealed
738 the ability of multi-omics to decipher MeOA in a non-target and non-model species, highlighting that
739 such promising approaches could help gain further insight into complex scenarios in relevant
740 environmental settings.

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748 **Appendix A. Supporting information**

749 Supplementary data associated with this article can be found in the online version.

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