Variations of biomarkers response in mussels *Mytilus galloprovincialis* to low, moderate and high concentrations of organic chemicals and metals

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Keywords: *Mytilus galloprovincialis*, contaminants, acetylcholinesterase, metallothioneins, catalase, lipid peroxidation

ABSTRACT

The changes of acethylcholinesterase activity (AChE), metallothioneins content (MTs), catalase activity (CAT) and lipid peroxidation (LPO) were assessed after 4 days exposure of mussels *Mytilus galloprovincialis* to a wide range of sublethal concentrations of chlorpyrifos (CHP, 0.03 – 100 µg/L), benzo(*a*)pyrene (B(*a*)P, 0.01 – 100 µg/L), cadmium (Cd, 0.2 – 200 µg/L) and copper (Cu, 0.2 – 100 µg/L). The activity of AChE in the gills decreased after

exposure to CHP and Cu, whereas no change of activity was detected after exposure to B(*a*)P and Cd. Both induction and decrease of MTs content in digestive gland occurred after exposure to CHP and B(*a*)P, while a marked increase was evident at highest exposure concentrations of Cd. The content of MTs progressively decreased of MTs with increasing concentration of Cu. CAT activity and LPO in the gills did not change after exposure to any of the chemicals. The results demonstrate different response profile in relation to the type of chemical compound, and highlight the potential implications for evaluation of biological effect of contaminants in marine environment. Furthermore, the AChE activity in the gills and MTs content in the digestive gland could be modulated by CHP and Cu at environmentally relevant concentrations indicating the potential risks of short-term transient mussels exposure that may occur due to run-off from land or accidental releases.

1. INTRODUCTION

Intensive anthropogenic activity along the coast represents the major source of contaminants that can be potentially harmful for the health of marine ecosystem. The adverse biological effect of these compounds has been evaluated by the measurement of early biochemical changes - biomarkers - in exposed marine organisms (McCarthy and Shugart, 1990). Mussels of genus *Mytilus* are among most commonly used sentinel organisms for monitoring of biological effect of various contaminants in marine environment, due to their sedentary lifestyle, expressed filter-feeding activity, capacity to accumulate and tolerate chemicals and wide geographical distribution (Widdows and Donkin, 1992).

Routine measurements of biomarkers related to important biological functions such as nerve impulse transmission, xenobiotic detoxification and antioxidant defence in mussels have nowadays been established as the basis for evaluation of marine waters quality in the risk assessment studies (Vidal-Liñán et al., 2010, 2014). For instance, the enzyme AChE involved

51 in cholinergic neurotransmission is frequently used as early biomarker of neurotoxicity in the
52 tissues of aquatic invertebrates due to its specific inhibition by organophosphate pesticides
53 (OP) (Campillo et al., 2013). Metalothioneins (MTs) are cysteine rich low molecular weight
54 cytosolic proteins, with high affinity to bind metals, and thus play crucial role in the
55 homeostasis of essential and detoxification of toxic metals (Viarengo et al., 1999). Evaluation
56 of total MTs content has been applied as biomarker of metal exposure in mussels
57 (Pytharopoulou et al., 2006). Organic and inorganic contaminants can act as pro-oxidants by
58 stimulating the generation of reactive oxygen species (ROS) in excess quantities thereby
59 provoking the onset of oxidative stress by disrupting the equilibrium maintained by
60 antioxidant mechanism of aerobic cell under normal physiological conditions (Regoli and
61 Giuliani, 2014). The enzyme catalase (CAT) has been among the constituents of the first line
62 of antioxidant defence due to its essential role in decomposition of hydrogen peroxide (H_2O_2),
63 a harmful by-product of cellular metabolism. This reaction represents the key strategy of
64 marine organisms for preventing complex reactions that might ultimately result in the cell
65 membranes lipid peroxidation (LPO) and consequently disruption of normal cellular function
66 (Regoli and Giuliani, 2014). Both CAT and LPO have been used as biomarkers of oxidative
67 stress in mussels, caused by exposure to various contaminants (Vidal-Liñán et al., 2010,
68 2013).
69 Previous studies also revealed that AChE, MTs, CAT and LPO could display limited
70 specificity and inconsistent response pattern depending on the intensity of stress and types of
71 marine contaminants that could potentially affect interpretation of biological effects in field
72 studies. Indeed, besides to OPs, modulation of AChE activity in mussels was detected
73 following exposure to polycyclic aromatic hydrocarbons (PAHs) and metals (Akcha et al.,
74 2000; Banni et al., 2010; Frasco et al., 2005; Kopecka-Pilarczyk, 2010). AChE inhibition was
75 also associated with metals and PAHs accumulation in the tissues of mussels deployed at

contaminated sites (Lehtonen et al., 2016; Vidal-Liñán et al., 2014). While the induction of MTs in mussels tissue due to cadmium (Cd) exposure is well documented, contradictory information is available regarding MTs response to copper (Cu), another metal of environmental concern (Maria and Bebianno, 2011; Perić et al., 2012; Pytharopoulou et al., 2011). Furthermore, MTs level in bivalves could be altered by the action of organic compounds (Maria and Bebianno, 2011; Schmidt et al., 2011). Conflicting responses of CAT and LPO were frequently recorded in bivalves after exposure to several contaminants (Amachree et al., 2013; Farkas et al., 2015; Maria and Bebianno, 2011; Ruiz et al., 2015). Inconsistent trends of both oxidative stress biomarkers were observed also in field exposed mussels despite significant accumulation of contaminants in their tissues (Rola et al., 2012; Vidal-Liñán et al., 2010).

Considering that response patterns of AChE, MTs, CAT and LPO in mussels to chemically different marine contaminants are not fully conclusive, is of interest to upgrade the existing background knowledge on the specificity and exposure - response relationships to wider range of chemicals. Thus, the aim of the present study was to determine the AChE, MTs, CAT and LPO variations after short-term low, medium and high intensity exposure to marine contaminants: (i) chlorpyrifos (CHP) as moderately persistent OP widely used in agriculture; (ii) benzo(*a)*pyrene (B(*a*)P) as model PAH combustion product; (iii) Cd, as toxic metal associated to industrial activity and (iv) Cu, a component of antifouling paints and pesticides. A wide range of exposure concentration was applied, including that occasionally found within polluted marine coastal areas or in case of spills and rain fall. These chemicals accumulate in mussels tissues within days (Durand et al., 2002; Geret et al., 2002; Gomes et al., 2012; Serrano et al., 1997; Zorita et al., 2007). The measurements of biomarkers were accompanied by chemical analyses performed in digestive gland tissue.

2. MATERIALS AND METHODS

2.1. Mussels handling and experimental setup

Adult mussels *Mytilus galloprovincialis* (shell length 60 – 70 mm) were purchased from aquaculture farm in Lim Bay and acclimated to laboratory conditions in tanks containing aerated and daily renewed seawater delivered by local seawater supply network from 10 m depth and 2 km distance from the coastline, for 7 days. Mussels were randomly distributed into 50 L polypropylene experimental tanks containing 42 animals (1 L/animal). The experiments were conducted for 4 days at 20 °C under semi-static conditions. Toxicants were renewed upon regular daily change of water. Mussels were not fed during acclimatisation and experiments. No mortality was recorded during that period.

The lowest exposure concentrations covered the annual average and maximum allowable concentrations in accordance with environmental quality standards for surface waters including coastal waters and territorial sea waters (European Commission, 2008). Intermediate concentrations occurred predominantly within polluted marine areas such as agricultural and urban areas, or following rain and accidental spills (Campillo et al., 2013; Pytharopoulou et al., 2006; Sanchez-Avila et al., 2013). The highest concentrations have been commonly applied in the studies of acute toxicity to induce the biomarker response. Mussels were exposed to nominal concentrations of: CHP (0 – control, 0.003% (V/V) of acetone – solvent control, 0.03, 0.1, 1, 5, 10 and 100 µg/L); B(*a*)P (0 - control, 0.003% (V/V) of dimethylsulphoxide (DMSO) – solvent control, 0.01, 0.05, 0.1, 1, 10 and 100 µg/L); Cd (as $CdCl_2$; 0 – control, 0.2, 1, 2.5, 10, 100 and 200 µg/L) and Cu (as $CuCl_2$; 0 – control, 0.2, 2, 10, 15, 20, 50 and 100 µg/L). Following exposure, digestive gland and gills tissues were removed from each organism, snap frozen in liquid nitrogen and stored as individual samples at -80 °C.

126 *2.2. Chemical analyses*

127 *2.2.1. CHP*

128 About 0.2 – 0.5 g of individual digestive glands samples were added with 25 mL of petrol
129 ether (Merck KGaA, Darmstadt, Germany) and 25 mL of dichloromethane (Suprasolv, Merck
130 KGaA, Darmstadt, Germany), extracted on a shaker for 1 hour. The obtained extracts were
131 filtered through cellulose filters (Filtres Fioroni, Ingre, France) purified on Florisil-bonded
132 phase cartridge and evaporated to dryness under nitrogen stream. Dry residuals were
133 dissolved in 2 mL of n-hexane (LiChrosolv, Merck KGaA, Darmstadt, Germany). An aliquot
134 of 1 µL was further processed by gas chromatograph coupled with mass detector Shimadzu
135 GC MS-QP2010Plus, equipped with autosampler Shimadzu AOC-5000 (Shimadzu, Kyoto,
136 Japan). Quantification of chlorpyrifos was performed using external standards (Restek,
137 Pennsylvania, USA) according to standardised protocols (EN 12393-1: 2013; EN 12393-2:
138 2013; EN 12393-3: 2008).

139

140 *2.2.2. B(a)P*

141 About 0.2 – 0.5 g of individual digestive glands samples were added with 25 mL of
142 cyclohexane (LiChrosolv, Merck KGaA, Darmstadt, Germany) and extracted on a shaker for
143 1 hour. The obtained extracts were filtered through glass fibre filter (MN GF-1, Macherey-
144 Nagel, Duren, Germany), purified on Florisil-bonded phase cartridge and evaporated to
145 dryness under nitrogen stream. The residuals were dissolved in 1 mL of methanol
146 (LiChrosolv, Merck KGaA, Darmstadt, Germany). An aliquot of 20 µL was further processed
147 by HPLC. The analysis was performed on Agilent 1260 Infinity system (Santa Clara,
148 California, USA) equipped with fluorescent detector (excitation 286 nm, emission 398 nm).
149 Quantification of B(*a*)P was performed using external standards (Restek, Pennsylvania, USA)
150 according to standardised protocols (EN ISO 15753: 2016).

151

152 *2.2.3. Cd and Cu*

153 About 0.5 g of individual digestive gland samples were digested using Anton Paar Multiwave
154 3000 microwave system (Perkin Elmer, USA) equipped with pressurized vessels, using 5 mL
155 of 65% nitric acid per sample (HNO3 Suprapur, Merck, Germany), over a 20 minutes
156 operation cycle at 200 °C. The digested samples were transferred to 25 mL volumetric flasks
157 and added with ultrapure water (Siemens). The concentrations of Cd and Cu were determined
158 using the graphite furnace atomic absorption spectrometer AAS800 equipped with S10
159 autosampler (Perkin Elmer, USA).
160 Analytical blanks were prepared and run in the same way as the samples. The concentrations
161 of metals were determined using external standards, with standard solutions prepared in the
162 same acid matrix. Standards for the instrument calibration were prepared on the basis of
163 single element standard solutions (LGC Standards, USA). The method for graphite furnace
164 AAS was validated using the IAEA-407 reference material (fish tissue), (International Atomic
165 Energy Agency, Austria). Mean recoveries for Cd and Cu were 91 and 90%, respectively. The
166 detection limits were: 0.0015 and 0.025 mg/kg for Cd and Cu, respectively.

167

168 *2.2.4. Measurement of biomarkers*

169 AChE activity was determined in gills tissue by the method of Bocquene and Galgani (1998).
170 Gills were homogenised in 0.02 M sodium phosphate buffer, pH 7.0, and centrifuged at
171 10000xg for 30 min at 4 °C. The reaction mixture consisted of 0.02 M sodium phosphate
172 buffer pH 7.0, 5.5'-dithiobis-2-dinitrobenzoic acid (0.5 mM final) and the appropriate amount
173 of gills' tissue sample. The absorbance increase at 415 nm was recorded every 30 seconds
174 after starting the enzymatic reaction with substrate acetylthiocholine (2.6 mM final). The
175 results were expressed as nmol thiocholine produced per min and per mg protein.

176 Concentration of MTs was determined in partially purified MTs extract from digestive gland
177 tissue according to the method of Viarengo et al (1999). Briefly, samples for MT analysis
178 were prepared by homogenisation of digestive glands pools in 20 mM Tris-HCl buffer, pH
179 8.6, 0.5M sucrose, containing 3 µg/mL Leupeptin , 90 µg/mL PMSF and 0.01% β-
180 mercaptoethanol, followed by acidic ethanol/chloroform fractionation. Standard curve of
181 reduced glutathione was used for quantification following spectrophotometric measurement
182 of absorbance at 412 nm. The results were expressed as ng MTs per g tissue (wet weight),
183 assuming mussels MTs molecular weight of 8600 Da and 21 cysteine residues per molecule.
184 Samples for oxidative stress parameters were prepared by homogenisation of gills tissue in
185 50mM K-phosphate buffer containing 2mM EDTA, pH 7.5, and centrifugation at 10000xg for
186 30 min at 4 °C. CAT activity was determined by kinetic measurement of hydrogen peroxide
187 (H_2O_2) decomposition at 25 °C (λ=240 nm) (Jamnik and Raspor, 2003). Results were
188 expressed as nmoles of H_2O_2 degraded per min and mg protein. LPO in the gills tissue was
189 assessed using the thiobarbituric reactive species assay (TBARS) (Bouskill et al., 2006) with
190 use of standard curve of 1,1,3,3-tetraethoxypropane. The absorbance was determined at 530
191 and 630 nm. After turbidity correction the values were expressed as nmol of malondialdehyde
192 (MDA) equivalents per mg of protein. Concentration of proteins in tissue samples was
193 detected using bovine serum albumin (BSA) as standard (Lowry et al, 1951).
194
195 *2.2.5. Data analysis*
196 Biomarkers data are presented as box and whisker plots, with square boxes indicating lower
197 and upper quartile and whiskers representing minimum and maximum data values (1.5
198 interquartile range). Median is depicted by solid line, and outliers as small circles.
199 Statistical analyses were performed using RStudio, version 0.98.1028 (RStudio Team, 2015).
200 Homoscedasticity and normality of data were checked by Levene's and Shapiro Wilk tests,

201 respectively. Significant differences among treatments were evaluated using one-way analysis
202 of variance (ANOVA), followed by Bonferroni post hoc test. When assumptions of
203 homogeneity of variance and normality were not accepted, the non-parametric Kruskal-Wallis
204 test was applied. When significant, the Mann Whitney's U test was applied to determine the
205 differences with respect to control. The significance level was set to $p<0.05$.
206
207 3. RESULTS
208 *3.1. Bioaccumulation of CHP, B(a)P, Cd and Cu in digestive gland*
209 All contaminants accumulated in *M. galloprovincialis* digestive gland in a concentration
210 dependent manner (Table 1). The accumulation of CHP was below 0.02 µg/g w.w. for
211 exposure concentrations from 0.03 to 1 µg/L while approximately three times higher values
212 were detected for exposure concentrations of 5 and 10 µg/L. The CHP uptake in digestive
213 gland tissue reached 1.7 µg/g w.w. in 100 µg/L - exposed mussels.
214 For exposure concentrations from 0.1 to 0.1 µg/L, the uptake of B(*a*)P was between 0.001 and
215 0.004 µg/g w.w. The accumulation of B(*a*)P markedly increased to 0.04, 0.3 and 2 µg/g w.w.,
216 for 1, 10 and 100 µg/L - exposed mussels, respectively.
217 Increased Cd accumulation was observed after exposure to 2.5 and higher Cd concentrations
218 (slightly above 1 and 3 µg/g w.w., respectively). Exposure to the highest Cd concentrations
219 (100 and 200 µg/L) resulted in notably increased accumulation to 24 and 29 µg/g w.w.,
220 respectively.
221 Cu accumulation displayed a slow gradual increase (1.27 to 2.44 µg/g w.w.) for exposure
222 concentrations between 0.2 and 20 µg/L. After exposure to 50 µg/L, accumulation of Cu
223 sharply increased while at 100 µg/L a slight decline was displayed (19.97 and 18.04 µg/g
224 w.w., respectively).
225

3.2. AChE activity in the gills

The effect of exposure to CHP, B(*a*)P, Cd and Cu on AChE activity in the gills of mussels is shown in Fig. 1. In general, a concentration - dependent decreasing trend in AChE activity was displayed after CHP exposure, with significant reduction with respect to control recorded at pesticide concentration of 0.1, 5, 10 and 100 µg/L. Exposure to B(*a*)P and Cd did not provoke significant changes in AChE activity. The activity of AChE after Cu exposure did not change at lower concentrations (0.2 - 10 µg/L), while significant reduction was recorded at intermediate and high concentrations (15 - 100 µg/L).

3.3. MTs concentration in the digestive gland

Concentration of MTs in digestive gland gradually increased at lower CHP concentrations and significantly higher MT level then control was recorded at 1 µg/L (Fig. 2). At higher pesticide concentration of 5, 10 and 100 µg/L, MTs concentration was not different than control. Following exposure of mussels to B(*a*)P, MTs concentration displayed an inconsistent trend. At exposure concentrations of 0.1 and 1 µg/L B(*a*)P, MTs concentration significantly decreased, whereas at 10 µg/L a notable and significant increase was recorded. MTs level was significantly reduced also after exposure to solvent control (DMSO) only. Exposure to Cd provoked a significant increase of MTs concentration at highest exposure concentrations (50 and 100 µg/L). At lower concentrations of Cd (0.2 – 10 µg/L), MTs level did not change. Gradual decrease of MTs level was recorded with increasing Cu concentrations, with significant difference with respect to control detected for exposure concentrations of 10 µg/L and higher.

3.4. CAT activity in the gills

250 CAT activity showed no significant difference between control and exposed group of mussels
251 for any chemical tested (Fig. 3).
252
253 *3.5. LPO in the gills*
254 No significant difference was found in MDA level between control and exposed mussels for
255 any chemical tested (Fig. 4).
256

257 4. DISCUSSION
258 A clear AChE activity reduction observed in the present study after CHP exposure is
259 consistent with previous reports of inhibitory effect on mussels AChE induced by this OP
260 compound (Dondero et al., 2011; Kopecka-Pilarczyk, 2010). However, it is noteworthy that in
261 the present study, mussels displayed a pronounced AChE inhibition at exposure to 0.1 µg/L
262 CHP. Similar environmentally relevant concentration of CHP induced disruption of the
263 mussels immune system (Patetsini et al., 2013). Therefore, as expected, the present results
264 infer that AChE represents a sensitive biomarker of CHP exposure in mussels. However, it
265 seems that similar assertion might not be applicable to bivalves in general, considering the
266 apparent insensitivity of *Ruditapes philippinarum* AChE after 7 days exposure to relatively
267 high CHP concentrations (12 and 24 µg/L) (Matozzo et al., 2006).
268 First evidence of B(*a*)P-induced neurotoxic effect in *M. galloprovincialis* and a strong
269 negative correlation of AChE activity in the gills and B(*a*)P accumulation in the tissues was
270 reported by Akcha et al (2000). However, the applied dose (50 mg B(*a*)P/kg d.w.) could be
271 considered relatively high since it corresponded to total PAHs concentration in the tissues of
272 mussels from contaminated French coast. Other literature data also suggest that PAHs could
273 inhibit AChE in the gills of mussels (Raftopoulou et al., 2006). In contrast, in the present
274 study the mussels gills AChE activity did not change after exposure to wide range of B(*a*)P

concentrations (0.01 - 100 µg/L). This clear lack of inhibitory effect on AChE could be related to differences in the exposure conditions and choice of tissue with respect to former studies. Indeed, AChE inhibition following acute exposure to 19 µg/L B(*a*)P was recently reported in *M. galloprovincialis* digestive gland (Kamel et al., 2012).

In agreement with previous reports for *Mytilus trossulus* (Pempkowiak et al., 2006) and *M. galloprovincialis* (Chalkiadaki et al., 2014), our results confirmed that AChE in mussels is not sensitive to Cd exposure *in vivo*, probably due to the well known efficient detoxification mechanism of Cd by MTs (Pytharopoulou et al., 2011).

Conversely, a significant concentration dependent reduction of AChE activity after exposure to midrange and high concentrations of Cu (15 – 100 µg/L) is in line with previous findings concerning short-term exposure to Cu concentrations of the same orders of magnitude in *M. galloprovincialis* (Regoli and Prinicipato, 1995) and *Ruditapes decussatus* (Hamza-Chaffaï et al., 1998). Our results are also consistent with studies reporting AChE sensitivity in *M. galloprovincialis* to Cu exposure in polluted marine environments (Vidal-Liñán et al., 2014). Interestingly, Cu concentrations that were comparable to those applied in the present study did not reduce AChE activity in *Mytilus edulis* after 7 days of exposure (Brown et al., 2004). Notwithstanding, AChE inhibition in the gills of the same species occurred at exposure to 40 µg/L Cu only after 7 days of exposure, whereas quicker inhibitory effect (after 3 days) was provoked at substantially higher metal concentration of 200 µg/L (Lehtonen and Leiniö, 2003). Besides, in the aforementioned study, Cu exposure did not provoke AChE activity reduction in the clam *Macoma balthica*. Similarly, AChE activity was not altered after 4 days exposure to 25 - 150 µg/L Cu in bivalve *Scrobicularia plana* (Bonnard et al., 2009). Thus, considering the present results and literature data, the Cu inhibitory effect on bivalves AChE seems to be species-specific, even for the same genus.

299 Alterations of MTs level in mussels digestive gland following exposure to organic compounds
300 and metals displayed strikingly different patterns. Previous studies provided evidences of
301 MTs ROS scavenging capability in bivalves *M. galloprovincialis* (Buico et al., 2008) and
302 *Cerastoderma edule* (Figueira et al., 2012). In this respect, MTs content rise observed at low
303 and significant effect at midrange concentration of CHP might indicate the prominent
304 requirements for scavenging of ROS generated by the action of pesticide that moderately
305 accumulated in the digestive gland. In fact, exposure to environmentally relevant CHP
306 concentrations (0.05 µg/L) induced ROS production in the mussels *M. galloprovincialis*
307 haemocytes (Patetsini et al., 2013). In addition, MTs content increase that strongly suggests
308 their involvement in detoxification of CHP-exposure generated ROS was reported for fish and
309 other invertebrates (Banni et al., 2011; Itziou and Dimitriadis, 2012). However, it is
310 interesting to note that substantial CHP bioaccumulation in the digestive gland at exposure
311 concentrations above 5 µg/L was not concomitant to increased MTs level. This nearly bi-
312 phasic pattern of MTs changes might imply the contribution of other components of
313 antioxidative defence in ROS neutralisation at higher CHP concentrations.
314 The response of MTs to B(*a*)P was fairly inconsistent. A significant reduction of MTs level in
315 the mussels digestive gland reported here was also previously observed following exposure to
316 B(*a*)P but at 10 times higher exposure concentration (10 µg/L) and longer exposure time (7
317 days) by Maria and Bebianno (2011). The effect observed in the aforementioned study was
318 explained by the specificities of biosynthetic pathways for antioxidant systems components
319 (that is, competition for cysteine residues needed for GSH synthesis). Conversely, in the
320 current study exposure to the equally high B(*a*)P concentration resulted in a strong induction
321 of MTs synthesis in mussels. This obvious discrepancy between the two studies could be
322 attributed to different exposure time (4 *vs* 7 days). Indeed, MTs content in mussels digestive
323 gland could significantly vary over a relatively short exposure time-frame of few days (Wang

et al., 2011). Nevertheless, the overall results obtained for MTs level changes after B(*a*)P exposure, should be regarded as inconclusive since weak, but significant MTs reduction occurred also for solvent control (DMSO). Hence, a rather aberrant MTs response to B(*a*)P exposure remains unclear, although a certain degree of sensitivity of MTs in digestive gland of *M. galloprovincialis* might again illustrate the MTs ability for scavenging of B(*a*)P - generated ROS. In fact, it was reported that B(*a*)P promote the production of ROS in mussels haemocytes (Gómez-Mendikute and Cajaraville, 2003).

Although the effect of B(*a*)P has been investigated in numerous studies, only few reports of its effect on MTs level are available for aquatic invertebrates, and only for relatively high concentration. However, modulation of MTs content seems to be more relevant upon exposure to mixtures containing B(*a*)P. In fact, co-exposure of B(*a*)P and metals might affect metal-binding properties and MTs induction in mussels with respect to single compounds only (Maria and Bebianno, 2011; Wang et al., 2011).

A significant MTs induction triggered at exposure to 100 and 200 µg/L Cd was consistent with the findings of marked total MTs protein increase achieved after short time exposure to Cd concentrations predominantly of the same order of magnitude (≥50 µg/L) (Marie et al., 2006; Pythralopoulou et al., 2011; Wang et al., 2011; Zorita et al., 2007). Conversely, MTs reduction occurred simultaneously with Cu accumulation in digestive gland. It is interesting to note the apparent lack of MTs content change in *Mytilus* digestive gland observed during acute exposure to relatively high Cu exposure concentrations of 50 and 100 µg/L (Brown et al., 2004; Lehtonen and Leiniö 2003; Maria and Bebianno, 2011; Pytharopoulou et al., 2011; Zorita et al., 2007). Furthermore, MTs content in mussels native to contaminated coastal site did not change with respect to reference site, despite significant accumulation of Cu in the tissues (Perić et al., 2012). Nearly opposite effect of Cd and Cu exposure on digestive gland MTs content in *M. galloprovincialis* could be explained by strong metal-specific up-

regulation of MT isoforms genes and concomitantly MTs expression at protein level (Banni et al., 2007; Dondero et al., 2005; Zorita et al., 2007). Accordingly, reduction of total cytosol MTs concentration could indicate higher efficiency of Cu-MTs complex breakdown with respect to induction of *de novo* MTs synthesis within such a short time frame (Serafim and Bebianno, 2009). The absence of MTs induction evident particularly at higher Cu exposure concentrations, may also suggest the "spill over" effect, that is, the surpassing of binding capacity for metals, resulting in decreased MTs sequestering ability and the onset of overall toxic effect (Le et al., 2016; Serafim and Bebianno, 2009).

The lack of detectable change of CAT activity and LPO in the gills of mussels to both organic chemicals and metals is apparently in contrast to previous reports reporting notable increase of both oxidative stress biomarkers after short term exposure to metals and B(*a*)P in mussels tissues (Akcha et al., 2000; Geret et al., 2002; Gomes et al., 2011, 2012; Maria and Bebianno, 2011; Rocha et al., 2015; Vlahogianni and Valavanidis, 2007). On the other hand, in line with our results, no change was observed of mussels gills CAT activity after Cu exposure (Farkas et al., 2015; Regoli and Principato, 1995; Ruiz et al., 2015) and LPO after Cd exposure (Amachree et al., 2013).

The exposure concentrations applied in the present study represent a significant pro-oxidant challenge by giving rise to ROS production and concomitant induction of antioxidant enzymes in mussels within short time (days) (Pytharopoulou et al., 2011). Thus, the absence of any detectable alterations of either CAT or LPO could be related to the fact that these oxidative biomarkers fluctuate considerably over time, and are not necessarily mutually coordinated (Amachree et al., 2013; Gomes et al., 2011; Vlahogianni and Valavanidis, 2007). For instance, the response of CAT often shows a distinct bi-phasic profile, with prominent increase at initial phase of exposure and a decline of activity with continuation of toxic insult

373 (Banni et al., 2010), that seems to account for lower CAT activity in mussels from chronically
374 contaminated sites, with respect to the reference (Rola et al., 2012).
375 Besides, these oxidative stress biomarkers quite differ between tissues (Amachree et al., 2013;
376 Maria and Bebianno, 2011; Regoli and Principato, 1995; Vlahogianni and Valavanidis, 2007).
377 For instance, regardless of the important role of gills as the first barrier to contaminants in the
378 surrounding environment, it seems that digestive gland display higher activity of CAT and
379 might be more appropriate for its measurement (Vidal-Liñán and Bellas, 2013).
380 Finally, the inability to distinguish a clear response pattern for oxidative stress parameters
381 could be linked to larger interindividual differences in the capacity of antioxidative machinery
382 for protection of lipid membranes. Besides, for each experiment a different mussels batch was
383 used from the same mussels raft. Thus, the basal levels variability observed in particular for
384 LPO might result from specificities of antioxidant system intrinsic to different mussels
385 batches. Lower interindividual variability and basal LPO, might indicate higher capacity of
386 Cu-exposed mussels for maintaining the balance between oxidant insult and antioxidant
387 defence.
388 An additional factor to consider is the involvement of other components of antioxidative
389 pathways that might have neutralised the generated ROS, although it is important to note that
390 they were not assessed within the frame of this study. In fact, superoxide dismutase (SOD) is
391 a primary antioxidant enzyme that decomposes superoxide anion and generates hydrogen
392 peroxide (H_2O_2) (Regoli and Giuliani, 2014). Besides CAT, H_2O_2 is also reduced to H_2O by
393 glutathione peroxidase (GPx), in association with GSH oxidation to GSSG, and reduction of
394 GSSG by glutathione reductase (GR) to maintain the GSH/GSSG ratio. Indeed, the important
395 contribution of GPx and phospholipid hydroperoxide GPx in maintenance of low MDA level
396 in mussels highlighted the protective role of these enzymes against potentially harmful
397 oxidative action of metals on lipid membranes (De Almeida et al., 2004). Additionally, when

oysters *Crassostrea gigas* were exposed to diesel oil at different salinity regimes, LPO remained unchanged despite consistent absence of CAT response (Zanette et al., 2011). The effective antioxidative response that prevented lipid peroxidation of membranes was indicated by a concomitant increase of glutathione-S-transferase activity that is important for conjugation and detoxification of potentially harmful organic compounds (Regoli and Giuliani, 2014).

Evidently, evaluation of the toxic impact of prooxidant - triggering compound in the risk assessment studies should not be confined to individual parameters of oxidative stress, due to transitory nature, complexity and tissue specificity of antioxidative response (Regoli and Giuliani, 2014). Furthermore, investigation of biochemical parameters simultaneously with more sensitive response on the level of gene expression, with emphasis on post-translational mechanisms of protein synthesis, has been suggested.

5. CONCLUSIONS

This study further corroborates the usefulness of AChE as sensitive biomarker of neurotoxicity in mussels *M. galloprovincialis* that is not restricted to OPs, since both CHP and Cu were capable of inducing a clear-cut reduction of AChE activity. Contrastingly, no such evidence could be obtained for B(*a*)P and Cd.

The opposite trends of digestive gland MTs level changes were displayed for Cd and Cu (increase and decline, respectively). That illustrates potential drawbacks in assessment of increased metal-detoxification requirements caused by metal exposure solely on the basis of total MTs concentration, as the measured value take into account the contribution of several MT isoforms. Due to insufficient clarity of response to CHP and B(*a*)P, the involvement of MTs in the process of ROS neutralisation require additional experimental evidences.

422 The fairly simple experimental design of short duration that includes measurement of
423 biomarkers response only at one point in time did not allow determination of the onset of
424 oxidative stress response, despite wide range of exposure concentrations. Thus, time
425 dependent studies that take into account the whole network of antioxidative parameters are
426 necessary to gain broader knowledge on the specificities of antioxidative defence in mussels.
427 Finally, a rapid and sensitive response reinforces the utility of AChE as reliable biomarker of
428 recent exposure to environmentally meaningful concentrations of potentially toxic chemicals.
429 In this respect, the results of the present study emphasize the potential threats for non-target
430 organisms, including mussels, which are likely to occur transiently following accidental spills,
431 sudden or seasonal runoff events within lagoons, estuaries and other marine coastal areas
432 characterised by slow exchange of water mass.
433
434 ACKNOWLEDGEMENTS:
435 This study was supported by the Ministry of Science and Education of the Republic of
436 Croatia.
437

438 LITERATURE

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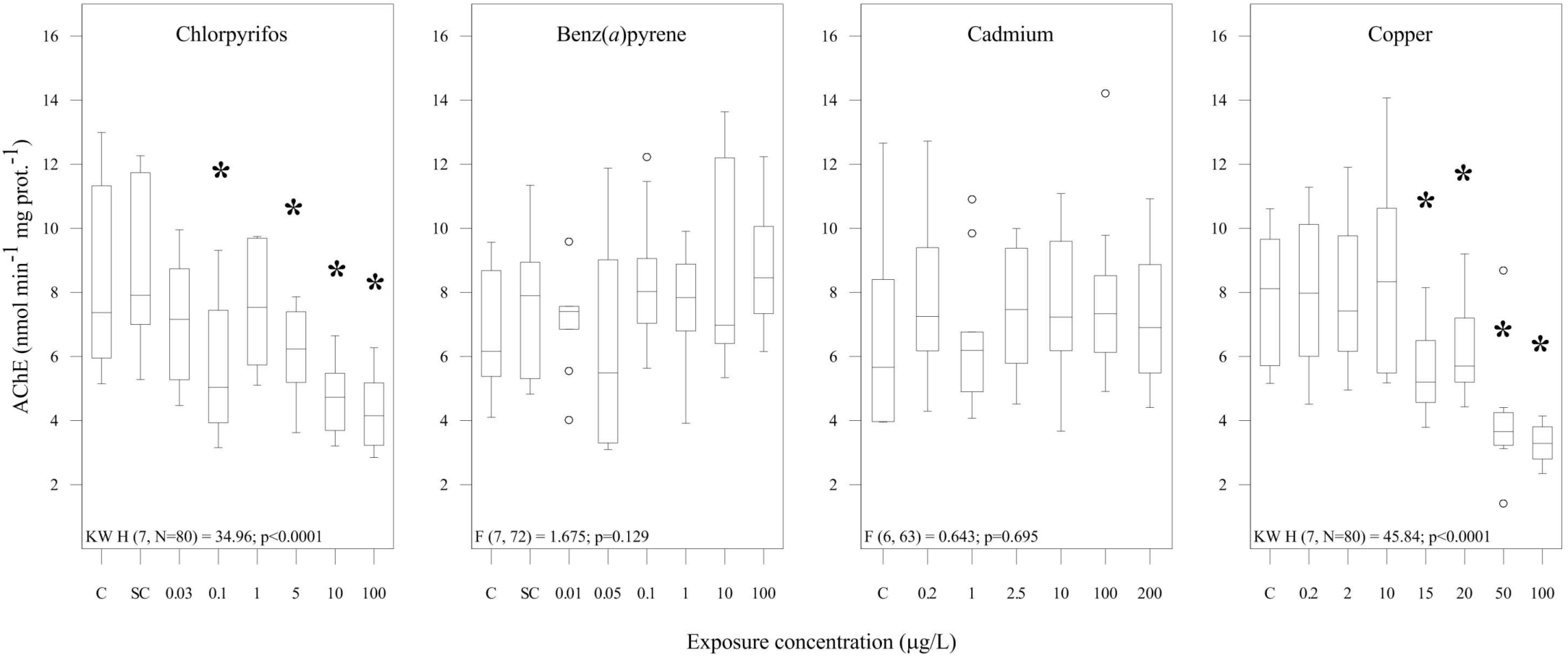
FIGURE CAPTIONS:

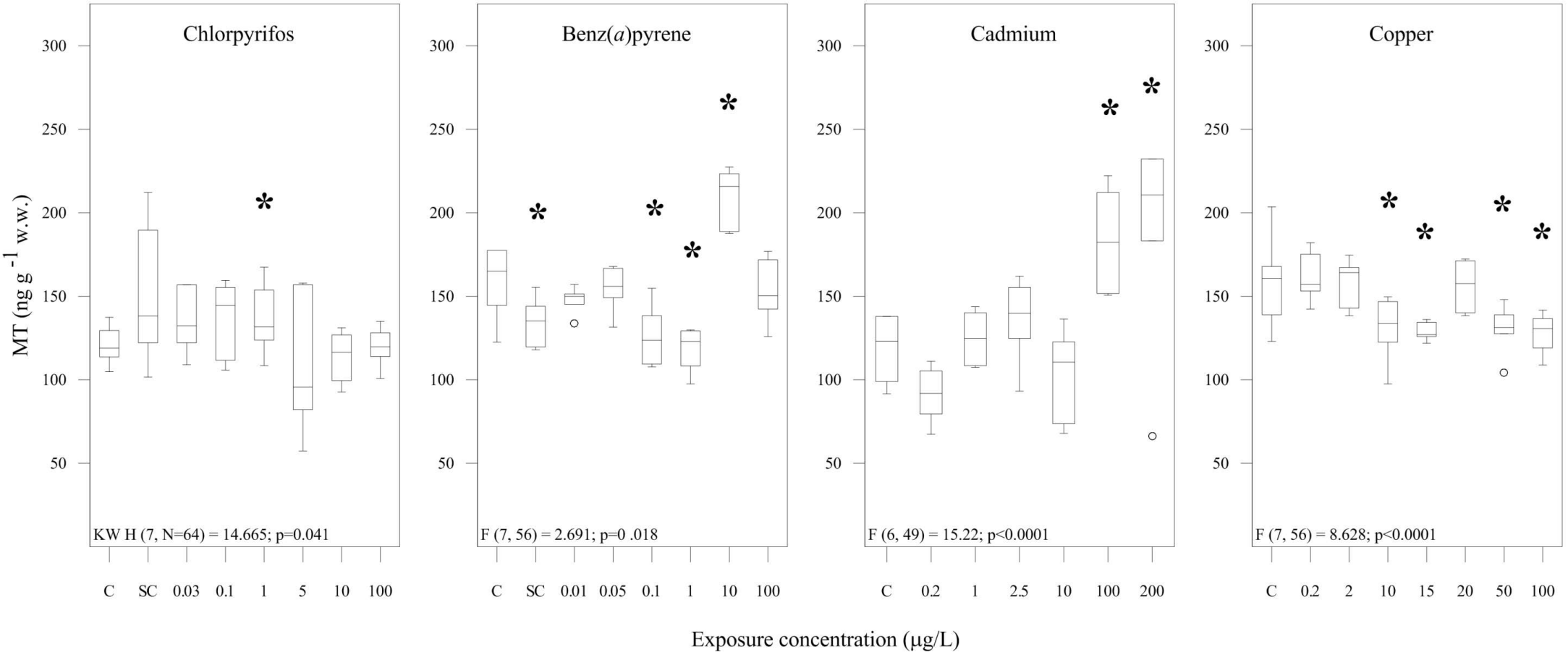
Figure 1. AChE activity (nmol min^{-1} mg $prot.^{-1}$) in the gills of mussels after exposure to a) CHP, b) B(*a*)P, c) Cd and d) Cu (n = 10 mussels for each contaminant); C, Control; SC, Solvent control; * Significant difference ($p<0.05$) with respect to C. See Section 2.2.5. for further details.

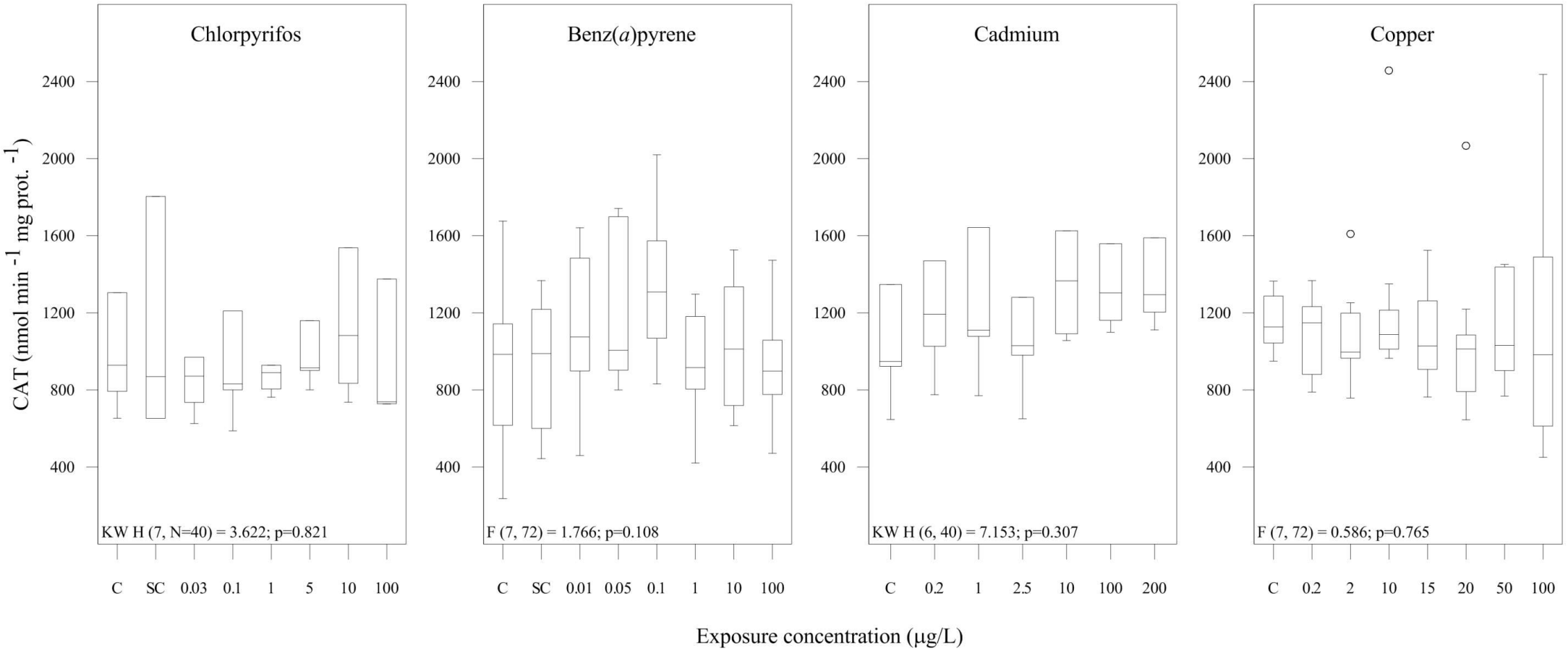
Figure 2. MTs concentration (ng g^{-1} w.w.) in digestive gland of mussels after exposure to a) CHP, b) B(*a*)P, c) Cd and d) Cu (n = 8 pools of 3 mussels for each contaminant); C, Control; SC, Solvent control; * Significant difference ($p<0.05$) with respect to C. See Section 2.2.5. for further details.

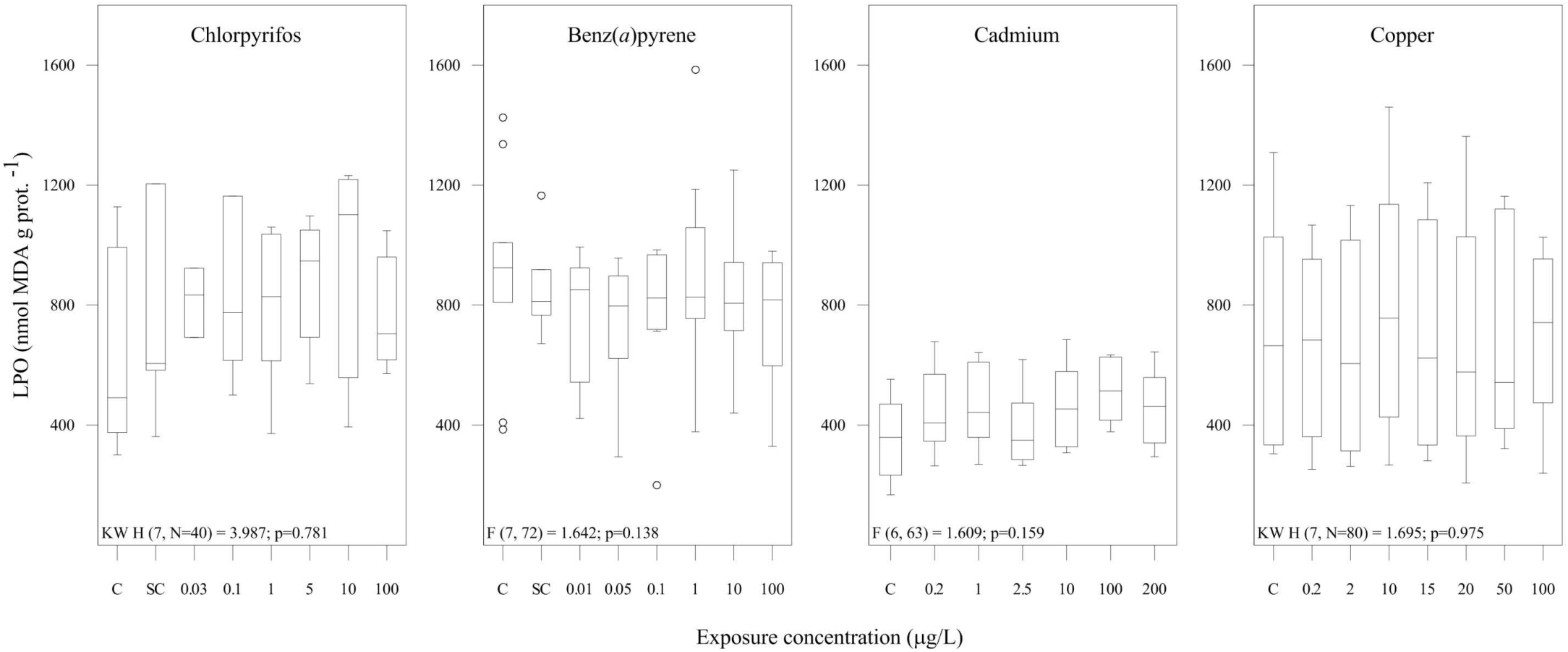
Figure 3. CAT activity (nmol min^{-1} mg $prot.^{-1}$) in the gills of mussels after exposure to a) CHP, b) B(*a*)P, c) Cd and d) Cu (n = 5 mussels for CHP and Cd, 10 for B(*a*)P and Cu); C, Control; SC, Solvent control; * Significant difference ($p<0.05$) with respect to C. See Section 2.2.5. for further details.

Figure 4. LPO (nmol MDA g $prot.^{-1}$) in the gills of mussels after exposure to a) CHP, b) B(*a*)P, c) Cd and d) Cu (n = 5 mussels for CHP, 10 for B(*a*)P, Cd and Cu); C, Control; SC, Solvent control; * Significant difference ($p<0.05$) with respect to C. See Section 2.2.5. for further details.


Chlorpyrifos
Benz(a)pyrene
Cadmium
Copper
AChE (nmol min-1 mg prot.-1)
KW H (7, N=80) = 34.96; p<0.0001
F (7, 72) = 1.675; p=0.129
F (6, 63) = 0.643; p=0.695
KW H (7, N=80) = 45.84; p<0.0001
C SC 0.03 0.1 1 5 10 100
C SC 0.01 0.05 0.1 1 10 100
C 0.2 1 2.5 10 100 200
C 0.2 2 10 15 20 50 100
Exposure concentration (µg/L)


Chlorpyrifos
Benz(a)pyrene
Cadmium
Copper
MT (ng g -1 w.w.)
300
250
200
150
100
50
KW H (7, N=64) = 14.665; p=0.041
F (7, 56) = 2.691; p=0 .018
F (6, 49) = 15.22; p<0.0001
F (7, 56) = 8.628; p<0.0001
C SC 0.03 0.1 1 5 10 100
C SC 0.01 0.05 0.1 1 10 100
C 0.2 1 2.5 10 100 200
C 0.2 2 10 15 20 50 100
Exposure concentration (µg/L)


Chlorpyrifos
Benz(a)pyrene
Cadmium
Copper
CAT (nmol min -1 mg prot. -1)
2400
2000
1600
1200
800
400
KW H (7, N=40) = 3.622; p=0.821
F (7, 72) = 1.766; p=0.108
KW H (6, 40) = 7.153; p=0.307
F (7, 72) = 0.586; p=0.765
C SC 0.03 0.1 1 5 10 100
C SC 0.01 0.05 0.1 1 10 100
C 0.2 1 2.5 10 100 200
C 0.2 2 10 15 20 50 100
Exposure concentration (µg/L)


Chlorpyrifos
Benz(a)pyrene
Cadmium
Copper
LPO (nmol MDA g prot. -1)
1600
1200
800
400
KW H (7, N=40) = 3.987; p=0.781
F (7, 72) = 1.642; p=0.138
F (6, 63) = 1.609; p=0.159
KW H (7, N=80) = 1.695; p=0.975
C SC 0.03 0.1 1 5 10 100
C SC 0.01 0.05 0.1 1 10 100
C 0.2 1 2.5 10 100 200
C 0.2 2 10 15 20 50 100
Exposure concentration (µg/L)