The effect of copper and chlorpyrifos co-exposure on biomarkers in marine mussels *Mytilus galloprovincialis*

Lorena Perić[a,*], Petra Burić[a,b]

[a] Ruđer Bošković Institute, Centre for Marine Research, Giordano Paliaga 5, 52210, Rovinj, Croatia

[b] Marine Sciences, Juraj Dobrila University of Pula, Pula, Croatia

*Corresponding author. Present address: Ruđer Bošković Institute, Division for Marine and Environmental Research, Bijenička cesta 54, 10000 Zagreb, Croatia.

Phone: 00 385 1 4680943

e-mail: Lorena.Peric@irb.hr

Abstract

Metals and organophosphorous pesticides commonly co-occur in marine environment, but the effect of their mixtures on non-target organisms is still poorly understood. This study investigated the combined effect of the essential metal copper (Cu) and organophosphorous pesticide chlorpyrifos (Chp) in mussels *Mytilus galloprovincialis* after short-term exposure to their sublethal concentrations. Mussels were exposed for four days to 5 and 15 μg l^{-1} Cu and 0.05 and 5 μg l^{-1} Chp, and to their binary mixtures. The investigated biomarkers, namely acetylcholinesterase activity (AChE), glutathione *S*-transferase activity (GST), metallothioneins content (MTs) and lipid peroxide levels (LPO) displayed unspecific and inconsistent response patterns that varied depending on the concentration of chemicals and composition of mixtures. The exposure to Cu or Chp alone did not induce AChE activity changes, whereas only Cu provoked a significant GST activity increase. Exposure to lower and higher concentration of Chp resulted in MTs content and LPO level increase, respectively. Response of biomarkers to mixtures was generally inconsistent. Data integration by IBR index and PCA revealed different stress levels for given exposure conditions, but no explicit differentiation between single and joint exposures was found. The present results showed that low and environmentally relevant concentrations of Cu and Chp in mixtures may result in a detectable biological response, stressing the need for further investigation of joint effects of widespread marine contaminants in sentinel organisms.

Keywords: mixture, metals, pesticides, integrated biomarker response, environmentally relevant concentrations

1. Introduction

The potentially harmful impact of chemicals released into marine environment has been extensively investigated over several decades. The majority of contaminants rarely occur as single compounds, and are most commonly present in the form of mixtures containing chemicals that either do not interact or mutually interact to exert joint actions. Thus, studies of their combined effect have become increasingly important for predicting toxic impact of contaminants on marine organisms (Maria and Bebianno, 2011; Ragusa et al., 2017; Roesijadi et al., 2009).

Of all the major contaminants of coastal ecosystems, metals have received significant attention given their ubiquitous distribution and long term persistence in the sediment and biota. Additionally, transient release of pesticides applied in agriculture and industry, either by accidental spillage, rain falls or run-offs, is likely to represent a threat for non-target organisms, particularly within estuaries, lagoons and other marine areas with slow water circulation.

Contamination by metals and pesticides has emerged as a serious worldwide concern for marine environmental health, but in general, the toxic effect of their mixtures has not been investigated at the same extent as of single compounds. Copper (Cu) is a naturally occurring trace metal essential for proper functioning of biological systems, but toxic when present in excess concentrations, as evidenced by numerous ecotoxicological studies reporting disruption of many biological functions in marine organisms (Cotou et al., 2012; Filimonova et al., 2016). Besides of being used as pesticide or fungicide, Cu is also incorporated as active ingredient in antifouling paints (Guardiola et al., 2012; Tornero and Hanke, 2016). Boat hulls leachates represent the main Cu source within the coastal zones, thereby greatly increasing the risk for marine non-target organisms (Ytreberg et al., 2010). Chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate; Chp) is a moderately persistent broad-spectrum organophosphorous pesticide, widely applied in agriculture. It mainly acts as inhibitor of acetylcholinesterase (AChE), a key enzyme of nerve signal transmission trough synaptic cleft,

but can also affect the immune and antioxidative system (Bertrand et al., 2016; Narra et al., 2017; Jin et al., 2015). Despite numerous pieces of evidence that point on negative effects of either Cu or Chp in aquatic organisms, studies addressing the combined effect of these two compounds are scarce (Tilton et al., 2011a, 2011b).

When organisms are subjected to chemical stressors, a range of molecular, biochemical and physiological changes may be quickly triggered at the sub-individual level. These sensitive early warning signals of potentially irreversible harmful effect that could occur at higher level of biological organisation are commonly known as biomarkers (Lagadic, 2002). The adverse sub lethal effect of contaminants has often been assessed by the use of biochemical biomarkers of neurotoxicity, detoxification and antioxidant defence. The enzyme acetylcholinesterase (AChE) is involved in cholinergic neurotransmission and is frequently used as early biomarker of neurotoxicity in aquatic invertebrates due to its specific inhibition by organophosphate pesticides and carbamates (Campillo et al., 2013). AChE is also sensitive to other organic compounds and metals (Akcha et al., 2000; Perić et al., 2017; Raftopoulou et al., 2006; Regoli and Principato, 1995). Glutathione *S*-transferase (GST) is involved in enzymatic biotransformation and elimination of a wide range of organic electrophilic compounds from the cells and reduction of lipoperoxides (Hayes et al., 2005). Thus, GST activity has been used as biomarker indicative of contaminant exposure (Campillo et al., 2013; Vidal-Liñán et al., 2014a). Metallothioneins (MTs) are low molecular weight proteins that bind metals with high affinity owing to their high cysteine content. These cytosolic proteins play a crucial role in the homeostasis of essential metals and detoxification of both toxic and essential metals in excess (Viarengo et al., 1999). Evaluation of total MTs content has been commonly used for assessment of metal stress in aquatic organisms (Maria and Bebianno, 2011; Perić et al., 2012). Organic and inorganic contaminants can act as pro-oxidants by stimulating generation of reactive oxygen species (ROS) over the level which is normally produced as by-product of

normal mitochondrial activity. These species may interact with and damage all types of biomolecules, such as lipids, resulting in a generation of lipid peroxides (LPO) and disruption of normal cellular function in marine organisms (Regoli and Giuliani, 2014). The LPO level increase as an indication of oxidative stress has been demonstrated in organisms from contaminated coastal areas (Banaoui et al., 2015; Benedetti et al., 2016; Maria and Bebianno, 2011; Jin et al., 2015).

The present study was aimed at investigating the short-term toxicity of Cu and Chp mixtures encompassing combinations of low and environmentally meaningful concentrations, in bivalve mussels *Mytilus galloprovincialis*. Mussels have long been used as sentinel organisms for ecotoxicological investigations owing to the expressed filter feeding activity, capability for accumulating and tolerating contaminants, sedentary lifestyle and widespread distribution (Widdows and Donkin, 1992). Toxic effect of Cu and Chp in mussels was evaluated by analysis of AChE activity, GST activity, MTs content and LPO levels. Biomarker data for different exposure conditions were synthesised using the integrated biomarker response (IBRv2; Beliaff and Burgeot, 2002; Sanchez et al., 2013) and principal component analysis (PCA).

2. Materials and methods

2.1. Experimental setup

Mussels *Mytilus galloprovincialis* of 60 – 70 mm shell length were purchased from local aquaculture facility. Prior to laboratory experiments, mussels were carefully displaced from socking and subsequently acclimated in tanks supplied by aerated seawater for 7 days. Mussels' specimens were randomly selected from acclimation tanks and distributed to adequate experimental polypropylene tanks (1 L/animal). Duration of exposure was 4 days and the experiment was conducted at 20 °C under semi-static conditions, and 16 h light: 8 h dark cycles.

Water in experimental tanks was changed every day, as well as the test solutions of toxicants. Mussels were not fed during the entire experiment and no dead animals were recorded.

Mussels were exposed to Cu (as $CuCl_2$; 5 and 15 μg L^{-1}), Chp (0.05 and 5 μg L^{-1}) and four combinations of Cu and Chp (Cu 5 μg L^{-1}/ Chp 0.05 μg L^{-1}, Cu 5 μg L^{-1}/ Chp 5 μg L^{-1}, Cu 15 μg L^{-1}/ Chp 0.05 μg L^{-1} and Cu 15 μg L^{-1}/ Chp 5 μg L^{-1}) and a solvent acetone (0.003 % final (v/v)). At the end of exposure, mussels were carefully opened to excise the digestive gland and gill tissue. Tissues were quickly frozen in liquid nitrogen and stored in cryovials as individual samples at -80°C until needed.

Selection of sub-lethal exposure concentrations was based on data relevant for marine coastal waters, in particular in case when significant quantities of contaminants from land-based sources may be delivered either directly, through run-offs and watercourses or via atmospheric deposition (Manfra and Accornero, 2005; Moreno-Gonzáles et al., 2013). Lower concentrations of Cu and Chp were within the range of annual average concentrations and below maximum allowable concentrations, respectively, in line with environmental quality standards in the field of water policy for surface waters (European Commission, 2008).

2.2. Measurement of biomarkers

For determination of AChE activity by the method of Bocquené and Galgani (1998), samples of gill tissue were individually homogenised in 0.02 M sodium phosphate buffer, pH 7.0 and the resulting homogenates were centrifuged at 10000 g for 30 min at 4 °C. The appropriate amount of gill tissue sample was added in the reaction mixture containing 0.02 M sodium phosphate buffer pH 7.0 and 5.5'-dithiobis-2-dinitrobenzoic acid (final concentration 0.5 mM)- The enzymatic reaction was then started by addition of substrate acetylthiocholine (final concentration 2.6 mM) and the absorbance increase at 415 nm was recorded every 30 seconds. The results were expressed as nmol of thiocholine produced per min and per mg of protein.

139 Content of MTs was determined in a partially purified low molecular weight fraction of
140 metalloproteins following acidic ethanol/chloroform extraction of digestive gland homogenate
141 (Viarengo et al. 1999). MTs were quantified after spectrophotometric measurement of
142 absorbance at 412 nm by using the standard curve of reduced glutathione. The content of MTs
143 was calculated by assuming the molecular weight of 8600 Da and 21 cysteine residues per
144 molecule. The results were expressed as µg MTs per g of tissue (wet weight).
145 Oxidative stress parameters were measured in the gill tissue sample prepared by
146 homogenization in 50mM K-phosphate buffer containing 2mM EDTA, pH 7.5, followed by
147 centrifugation at 10000 g for 30 min at 4 °C. Glutathione *S*-transferase (GST; EC 2.5.1.18)
148 activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig et al.
149 1974). The reaction mixture consisted of 0.1 M K-phosphate buffer pH 6.5, 1mM CDNB and
150 1 mM glutathione (GSH; $\varepsilon = 9.6\ mM^{-1}cm^{-1}$). The enzymatic reaction was monitored at 340 nm,
151 for 5 min. Activity of GST was expressed as nmol of CDNB conjugate produced per min and
152 per mg of protein. LPO level was assessed by thiobarbituric reactive species (TBARS) assay
153 (Buege and Aust, 1978) with the use of standard curve of 1,1,3,3-tetramethoxypropane. The
154 absorbance was determined at 530 nm and for turbidity correction at 630 nm. Levels of LPO
155 were expressed as nmol of malondialdehyde (MDA) equivalents per mg of protein.
156 Concentration of proteins in samples used for enzymatic analyses was determined by the
157 method of Bradford (1976) with the use of bovine serum albumin (BSA) as standard.
158
159 2.3. Data analysis
160 In order to integrate all biomarker data (AChE, GST, MT, LPO) into a general stress index, a
161 method for calculating the Integrated Biomarker Response (IBRv2) based on reference
162 deviations concept was applied, as originally described by Beliaeff and Burgeot (2002) and
163 modified by Sanchez et al. (2013). The control group was considered as reference. The IBRv2

index was calculated for each exposure condition by addition of deviation indices of each biomarker. Biomarker deviation indices for each exposure condition were reported in star plots. The areas above and below 0 indicate biomarker induction and inhibition, respectively (Beliaeff and Burgeot, 2002; Sanchez et al., 2013). Principal component analyses (PCA) was also used to visualise the biomarker responses for all exposure conditions.

The open source software RStudio, version 1.0.153 (RStudio Team, 2017) was used for all statistical analyses. The Levene's and Shapiro Wilk tests were used to check homoscedasticity and normality of data, respectively. One-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* test, were used to detect significant differences among treatments. When the requirements for homogeneity of variance and normality were not fulfilled, data were either log-transformed to achieve normality or non-parametric Kruskal-Wallis test was applied. I the latter case, differences with respect to control were determined by Dunn's test. The significance level was set to $p < 0.05$.

3. Results

Neither Cu nor Chp modulated AChE activity in mussels gill (Fig. 1). A significant decrease of AChE activity was detected for a mixture of higher concentrations of Cu (15 µg l^{-1}) and Chp (5 µg l^{-1}). Exposure to the mixture of lower concentrations of Cu and Chp (5 µg l^{-1} and 0.05 µg l^{-1}, respectively) resulted in a significant increase of AChE activity with respect to control.

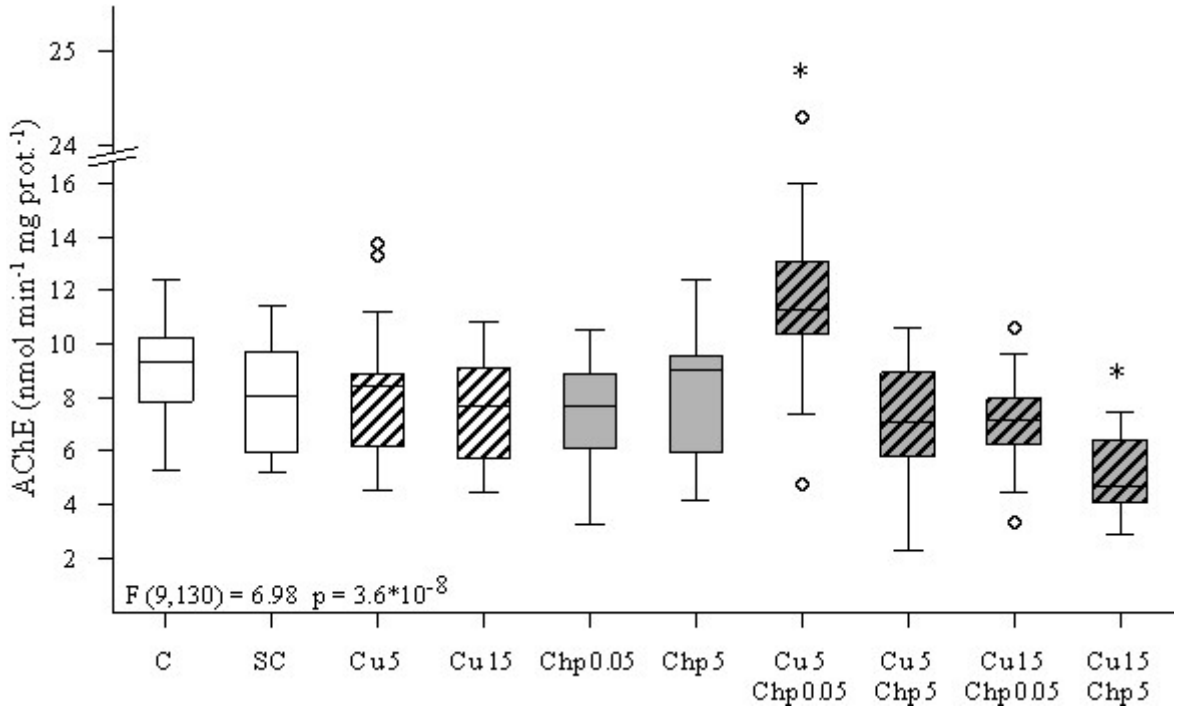

Figure 1. AChE activity (nmol min^{-1} mg $prot.^{-1}$) in the gill of mussels after exposure to Cu, Chp and their mixtures. C, Control; SC, Solvent control; * Significant difference ($p<0.05$) with respect to C.

As shown in Fig. 2, the activity of GST increased after Cu exposure in a concentration dependent manner, and a significant difference with respect to control was detected for both concentrations (5 and 15 µg l^{-1}). There was no effect of Chp on GST activity. Lower concentration of Chp combined with Cu significantly increased GST activity. A mixture of higher Chp concentration and Cu, had as well an inductive effect on GST activity, but that values were no longer significantly different from the control.

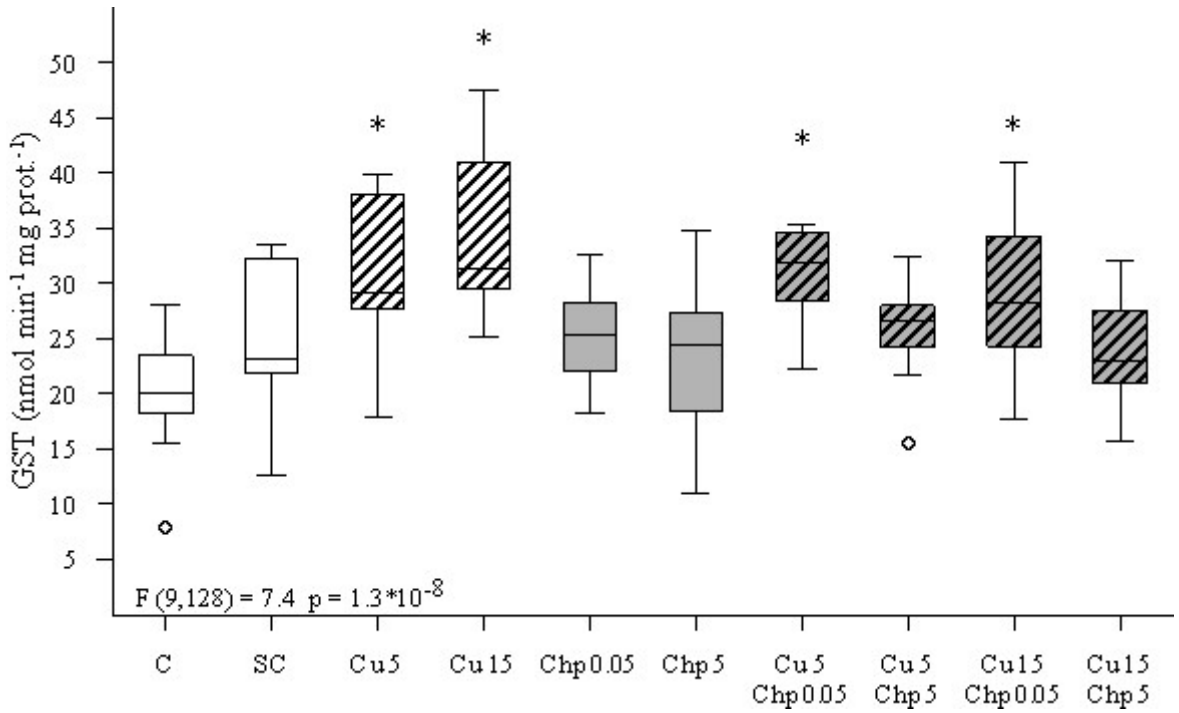

Figure 2. GST activity (nmol min^{-1} mg $prot.^{-1}$) in the gill of mussels after exposure to Cu, Chp and their mixtures. C, Control; SC, Solvent control; * Significant difference ($p<0.05$) with respect to C.

The content of MTs was significantly higher than control when mussels were exposed to Chp at lower concentration only (0.05 µg l^{-1}) whereas no effect could be observed for Cu at both concentration tested (Fig. 3). There was no effect on MT content of any of the four Chp and Cu combination.

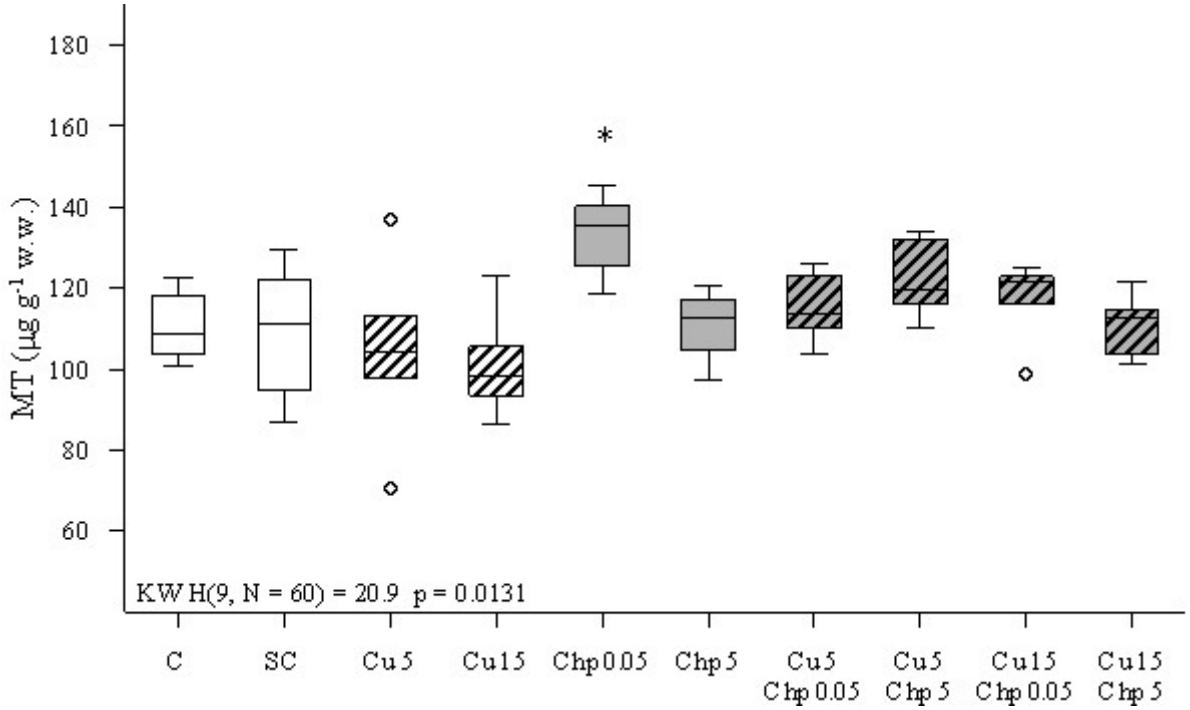

Figure 3. MT content (µg g^{-1} w.w.) in the digestive gland of mussels after exposure to Cu, Chp and their mixtures. C, Control; SC, Solvent control; * Significant difference ($p<0.05$) with respect to C.

With exception of mussels exposed to 5 µg l^{-1} Chp, a significant increase of LPO with respect to control was generally not observed (Fig. 4). Exposure to higher concentration of Cu (15 µg l^{-1}), alone or in combination with Chp, resulted in a significant decrease of LPO.

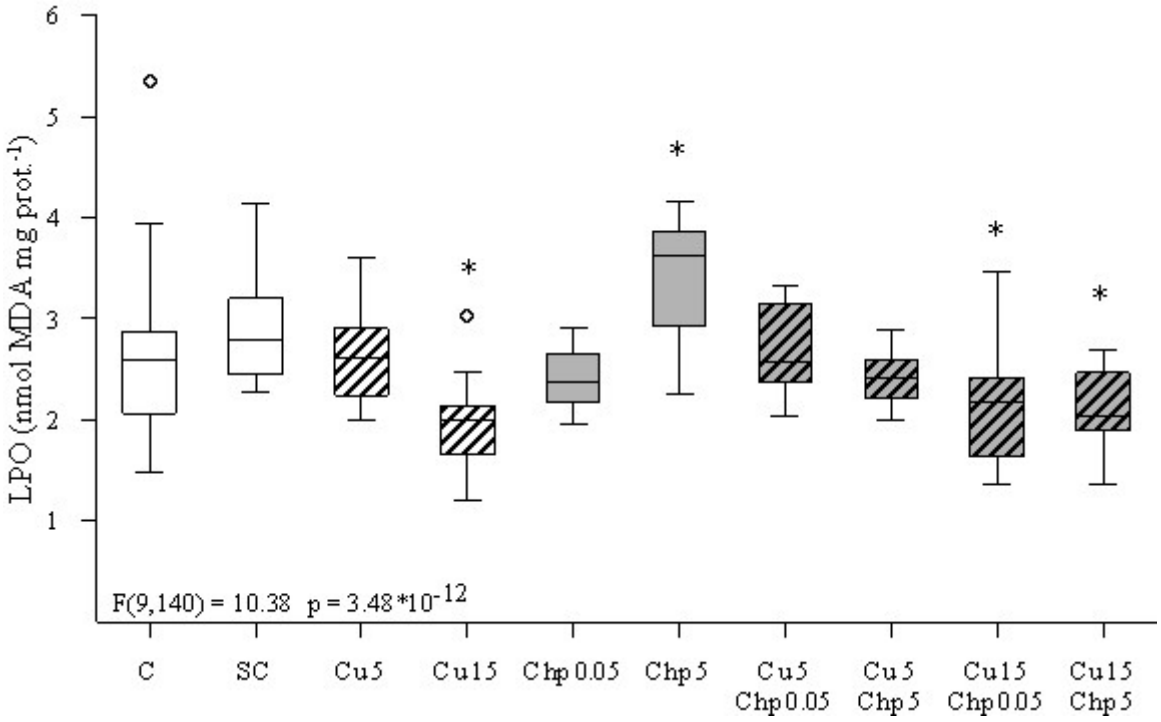

Figure 4. LPO level (nmol min^{-1} mg $prot.^{-1}$) in the gill of mussels after exposure to Cu and Chp individually and their mixtures. C, Control; SC, Solvent control; * Significant difference ($p<0.05$) with respect to C.

Integration of biomarker responses was performed using the IBRv2 index (Fig. 5). The contribution of each of the four biomarkers to the IBRv2 calculation differed between exposure conditions. The highest IBRv2 value was observed when mussels were exposed to 15 µg L^{-1} Cu. The order of exposure conditions in accordance to IBRv2 values was: Cu 15 µg L^{-1} > Cu 15 µg L^{-1} Chp 0.05 µg L^{-1} > Cu 15 µg L^{-1} Chp 5 µg L^{-1} > Chp 0.05 µg L^{-1}> Cu 5 µg L^{-1} Chp 5 µg L^{-1} > Cu 5 µg L^{-1} Chp 0.05 µg L^{-1} > Cu 5 µg L^{-1} > Chp 5 µg L^{-1}> SC (Fig. 5).

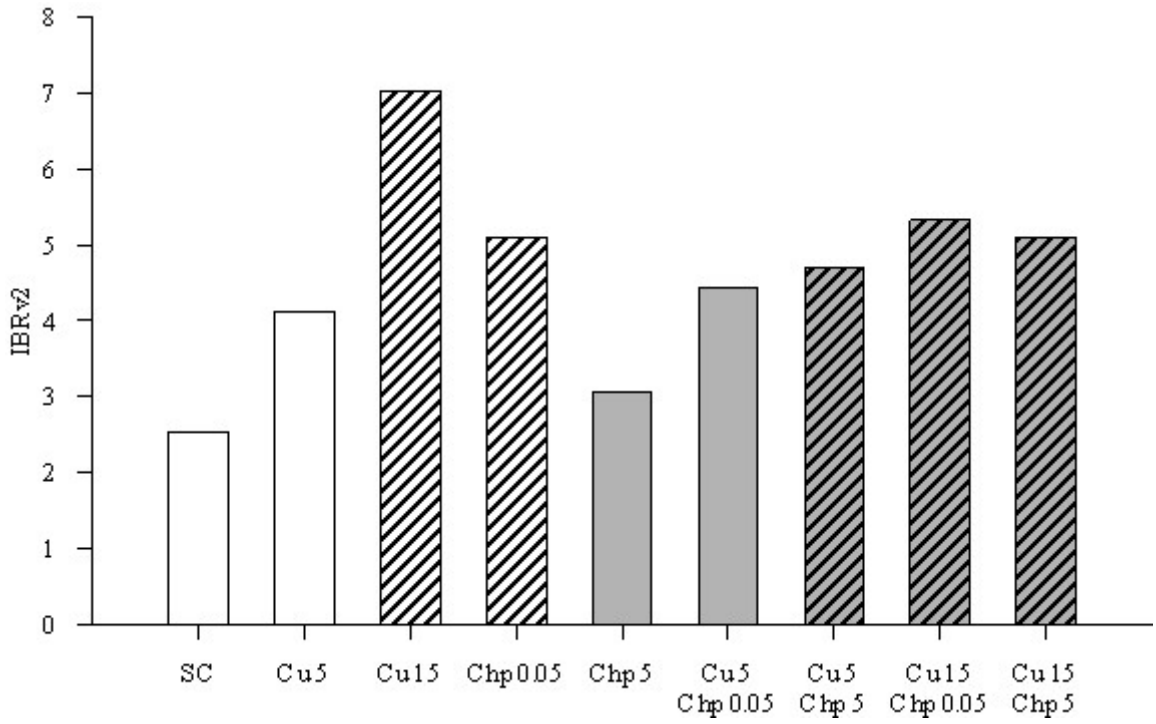

Figure 5. Integrated biomarker response index (IBRv2) summarizing the values for AChE activity, GST activity, MTs content and LPO level after exposure to Cu, Chp and their mixtures. C, Control; SC, Solvent control;

For each exposure condition, the investigated biomarkers displayed either increased or decreased values of deviation indices with respect to 0 value, and the sensitivity of biomarker responses could be visualised in the corresponding star plots (Fig. 6). The standardized values of biomarkers indicated that changes of GST activity represented the most sensitive response for the majority of exposure conditions. The increase of MTs content, LPO level and AChE inhibition displayed the highest deviation from 0 value at 0.05 µg L^{-1} Chp, 5 µg L^{-1} Chp and mixture of 15 µg L^{-1} Cu and µg L^{-1} Chp, respectively.

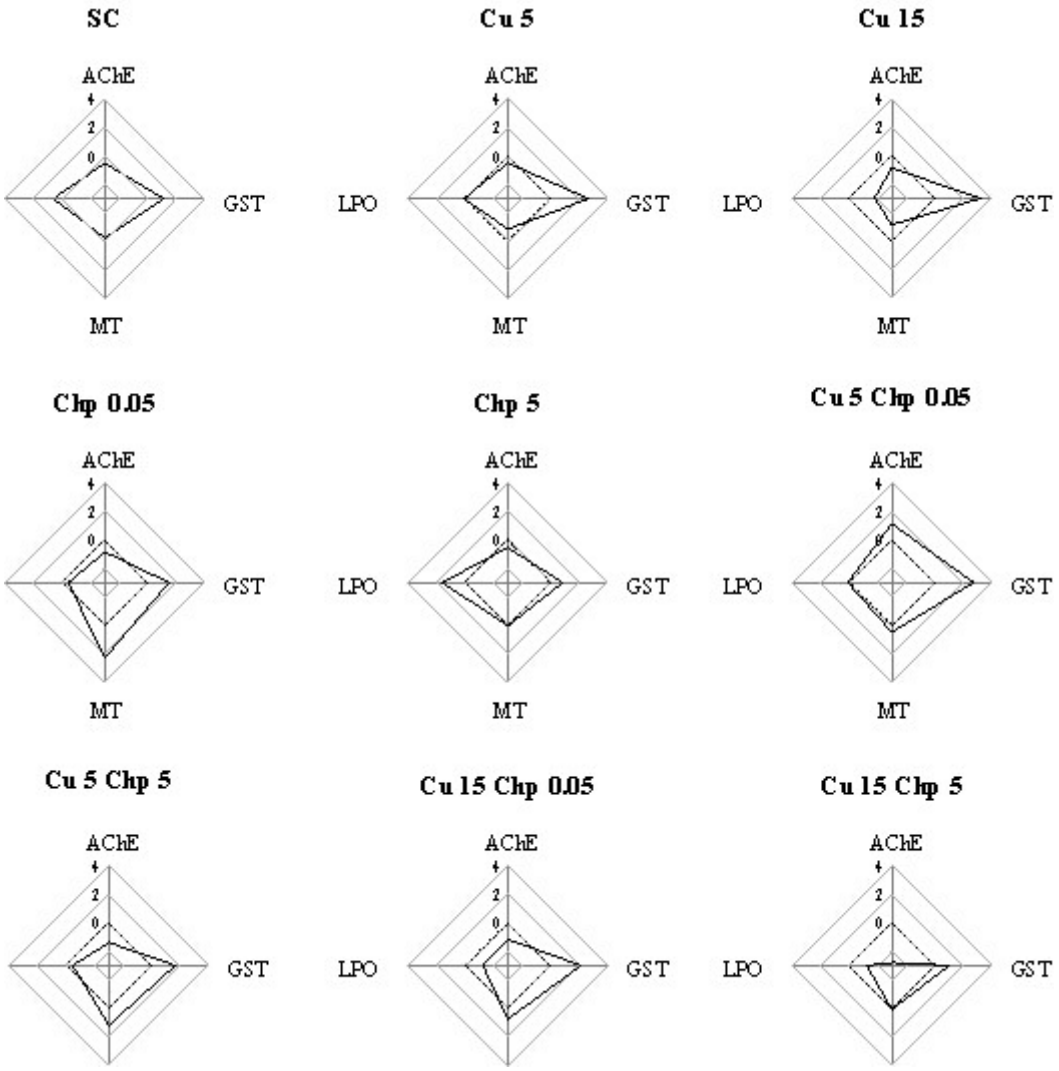

Figure 6. Deviation indices of AChE activity, GST activity, MTs content and LPO level in mussels *M. galloprovincialis* exposed to Cu, Chp and their mixtures. The dotted line depicts the control (reference) group. Solid line represents exposed groups (SC, Solvent control; Cu and Chp individually and in mixtures). Values above and below the dotted line correspond to induction and inhibition of biomarkers, respectively.

The Principal Component Analysis (PCA) clearly showed a spatial differentiation between different exposure conditions (Fig. 7). PC1 accounted for 39.23 % of variance and displayed a grouping of control, solvent control and mussels exposed to 5 μg L^{-1} Chp at the negative part of the axis, opposite to mussels exposed to a mixture of Cu (15 μg L^{-1}) and Chp (0.05 μg L^{-1})

that was associated mainly with a decrease of LPO content. PC2 that accounted for 34.7% of variance, separated two groups of mussels, each at the opposite side of the axis. Mussels exposed to Cu only, and to a mixture of Cu and Chp at lower concentrations, were associated with the positive part of axis PC2, and presented higher GST and AChE activity. Higher MTs content was a feature of mussels exposed to lower Chp concentration (0.05 μg L^{-1}) and to both mixtures of Chp at higher concentration (5 μg L^{-1}) with Cu.

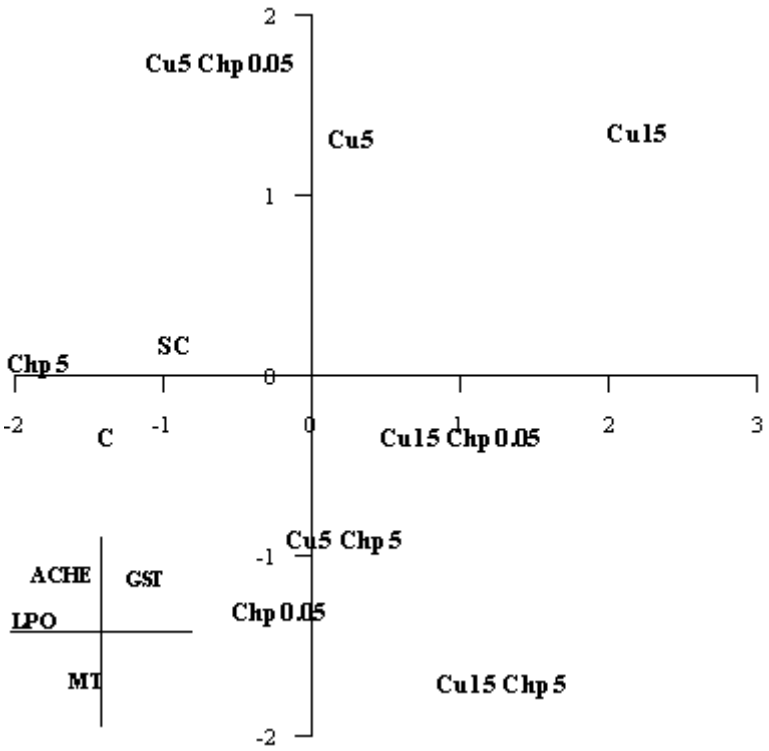

Figure 7. PCA bi-plot of biomarkers AChE activity, GST activity, MTs content and LPO level in mussels *Mytilus galloprovincialis* displaying the loadings of the variables and data scores as exposure conditions (C, Control; SC, Solvent control; Cu and Chp individually and in mixtures).

4. Discussion

The biomarkers studied in *M. galloprovincialis* were altered either by individual compounds only (MTs content), by mixtures only (AChE activity) or by both the individual compounds

and their mixtures (GST activity and LPO level). However, the response pattern of each biomarker was rather inconsistent and dependent on the concentrations of contaminants and mixture compositions.

Inhibition of AChE activity can occur at low and environmentally relevant concentrations of chemical contaminants, including Cu and Chp (Perić et al, 2017). Although only a slight and statistically not significant decreasing trend of AChE activity in the gill of mussels was recorded after single Cu and Chp exposures, the combination of these two compounds at their higher concentrations resulted in an expressed AChE activity inhibition. Similar joint neurotoxic effect of metals and pesticides was reported for marine copepods *Tigriopus brevicornis* (Forget et al., 1999) and mussels *Mytilus edulis* (Lehtonen and Leiniö, 2003). The combination of lower concentrations of Cu and Chp resulted in an opposite effect on AChE activity suggesting the occurrence of a hormetic response. Hormesis is a widely described biological phenomenon characterised by stimulatory and inhibitory response at low and high concentrations, respectively, of various toxicants in a large number of species (Calabrese and Blain, 2011). Stimulation at low doses of toxicants has generally been considered as beneficiary for organisms in terms of activation of various defence pathways and improved capability to maintain homeostasis (Calabrese, 2013). Yet, the occurrence of hormetic effect of contaminants on AChE activity has been reported only occasionally for invertebrates (Li and Tan, 2011; McHenery et al., 1997; Velki and Hackenberger, 2012). Therefore, more studies focused on the effect of low, environmentally meaningful concentrations of chemical stressors and in particular their mixtures are required to unravel the stress-related compensatory AChE response in mussels.

Transition metals such as Cu represent a potent catalyst of Fenton reaction, which generates toxic hydroxyl free radicals capable of oxidising a wide range of biological molecules including lipids in the cell membrane. This process may eventually lead to formation of LPO and

292 impairment of membrane function (Halliwell and Gutteridge, 2006). Exposure of *M.*
293 *galloprovincialis* to Cu alone did not result in LPO level increase although a strong pro-oxidant
294 activity of Cu was previously reported for bivalves (Jorge et al, 2018; Katsumiti et al., 2018).
295 The lack of LPO increase could be related to antioxidant function of GST that concurrently
296 displayed a concentration dependent increase of activity in response to Cu. GST is a non-
297 specific enzyme that could act as non-selenium dependent glutathion peroxidase by reduction
298 of LPO to alcohol, concomitantly with the oxidation of GSH to GSSG (Regoli and Giuliani,
299 2014). In agreement with our results, the capability of Cu to induce GST was demonstrated in
300 laboratory exposure experiments with *M. galloprovincialis* (Canesi et al., 1999) and *Perna*
301 *viridis* (Goswami et al., 2014). Nevertheless, in another study the GST activity did not change
302 when mussels *M. galloprovincialis* were exposed to a similar range of Cu concentrations for 7
303 days (Maria and Bebianno, 2011) although the inductive effect of Cu could be observed at the
304 level of GST protein expression (Maria et al., 2013). Noteworthy, increased GST activity was
305 also reported for bivalves from metal-polluted marine environments (Fernández et al., 2010;
306 Liu and Wang, 2016; Vidal-Liñán et al., 2014a).
307 The activity of GST has primarily been linked to its role as phase II detoxification enzyme in
308 conjugation of reduced glutathione (GSH) to a wide range of organic electrophilic compounds,
309 thereby transforming them to less toxic and water soluble compounds that are readily removed
310 from the cell (Hayes et al., 2005). Chp is activated by the cytochrome P-450 mediated oxidative
311 desulfurization, and the emerging oxons are further metabolised by GST during phase II drug
312 metabolism (Fujioka and Casida, 2007). In that respect, the increase of GST activity in some
313 invertebrates was associated to organophosphorous pesticide exposure, as evidenced either by
314 laboratory exposure experiments (Antognelli et al., 2006; Bertrand et al., 2016; Cacciatore et
315 al., 2015) or by analyses of field samples (Campillo et al., 2013). Nevertheless, other studies
316 reported inconsistencies or even the lack of GST activity changes in the tissues of aquatic

317 organisms exposed to this particular class of xenobiotics (Banaoui et al., 2015; Bianco et al.,
318 2013; Bonifacio et al., 2017). Accordingly, no significant changes occurred in the present study
319 after exposure to Chp only, thus failing to clearly display the GST involvement in detoxification
320 of Chp in *M. galloprovincialis*. The lack of a clear-cut GST response over wider time frame
321 and detoxification inefficiency of GST were also observed when mussels were exposed to other
322 classes of organic contaminants (Vidal-Liñán et al., 2014b, 2016).
323 The increase of LPO level after exposure to higher concentration of Chp was consistent with
324 previous studies showing an oxidative damage of lipid membranes in aquatic invertebrates
325 provoked by organophosphorous pesticides (Bertrand et al., 2016; Cacciatore et al., 2015).
326 Apparently, the action of antioxidative response components were more efficient at relatively
327 low Chp concentrations. Moreover, the results suggests that at lower Chp concentration, ROS
328 detoxification could be in part accounted for by scavenging action of MTs (Buico et al., 2008;
329 Itziou and Dimitriadis, 2012; Perić et al., 2017).
330 No changes of GST activity occurred in the present study after exposure to Cu mixed with
331 higher concentration of Chp. This lack of GST induction that was otherwise detected after
332 exposure to Cu only or when Cu was combined with lower Chp concentration could be
333 indicative of antagonistic effect that relies on an unknown mechanism of Cu and Chp
334 interaction. Furthermore, the absence of GST activity alterations did not coincide with LPO
335 level increase. Preservation of cell membranes integrity suggests an overall antioxidant defence
336 efficacy in elimination of radicals in mussels exposed to a mixture of environmentally relevant
337 concentrations of Cu and Chp. The observed variable patterns of LPO level could be explained
338 by an uncoordinated concentration and time-dependent action of ROS scavenging enzymes,
339 such as superoxide dismutase (SOD) that catalyse the decomposition of superoxide anion to
340 H_2O_2 as well as catalase (CAT) and glutathione peroxidase (GPx) that reduce H_2O_2 to H_2O
341 (Benedetti et al., 2016; Regoli and Giuliani, 2014). These enzymes are also involved in the

antioxidative defence of mussels exposed to metals and organophosphorous pesticides (Banaoui et al., 2015; De Almeida et al., 2004; Fang et al., 2010; Rocha et al., 2015).

In the present study, MTs content in the digestive gland was not altered by Cu exposure indicating that at applied concentrations and within a short time span, the basal physiological MTs amount was sufficient to sequester the excess of Cu ions and that degradation of Cu-MTs complex was more efficient than the *de novo* synthesis of MTs (Serafim and Bebianno, 2009). Likewise, the lack or even reduction of MTs content in mussels after Cu exposure were reported previously (Maria and Bebianno, 2011; Perić et al., 2017).

The integrated biomarker response (IBRv2) approach has been successfully applied for synthesis of biological responses to contaminants in various field and laboratory studies mostly reporting consistent relationships between IBRv2 values and contaminants level or exposure conditions (Vieira et al., 2016, 2018). In the present study, the IBRv2 discriminated the exposure conditions based on the response of biomarkers. However, the differences in the IBRv2 values between single compounds and mixtures were difficult to interpret, and the variable patterns of biomarker responses were further evidenced by graphical representation of standardised values of biomarkers in the star plots. Similarly, data integration using PCA analysis did not reveal a clear association of the intensity of biomarker responses and the severity of exposure conditions. The results also indicate that IBRv2 index as an indication of exposure risk could be misleading and susceptible to under or over estimation. For instance, exposure of mussels to 5 µg L^{-1} Chp resulted in low IBRv2 value, although significant increase of LPO content clearly indicated the occurrence of oxidative stress damage. Conversely, high IBRv2 recorded at 15 µg L^{-1} of Cu reflects the ability of mussels to endure the oxidative stress challenge by activating GST. This highlights the need for careful data interpretation, particularly considering possible mutual interference of mixture components, often

366 asynchronous and complex biomarkers responses and magnitude of response of each biomarker
367 (Quintaneiro et al., 2015).
368
369 5. Conclusions
370 The effect of short-term and environmentally realistic exposure to Cu and Chp mixtures was
371 not entirely straight-forward and varied in relation to concentrations of compounds. The activity
372 of GST was the most sensitive biomarker that revealed an oxidative challenge imposed by both
373 the single compounds and their mixtures. A clear biomarker response pattern that could be
374 attributed to mixtures could not be discerned, further stressing the challenges of stress response
375 evaluation in natural environment. It is necessary to highlight that the results of the current
376 study represent only a single capture of biomarker response profile at one point in time. Thus,
377 the investigation of temporal pattern based on biomarker data obtained over a wider time frame
378 is required for a clearer picture of the toxic effect of mixtures in sentinel organisms.
379
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