Ecotoxicological potential of non-steroidal anti-inflammatory drugs (NSAIDs) in marine organisms: Bioavailability, biomarkers and natural occurrence in Mytilus galloprovincialis

Ecotoxicological potential of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in marine organisms: bioavailability, biomarkers and natural occurrence in *Mytilus galloprovincialis*

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Abstract

Pharmaceuticals represent a major environmental concern since the knowledge on their occurrence, distribution and ecotoxicological potential is still limited particularly in coastal areas. In this study, bioaccumulation and cellular effects of various non steroidal anti-inflammatory drugs (NSAIDs) were investigated in mussels *Mytilus galloprovincialis* to reveal whether common molecules belonging to the same therapeutic class might cause different effects on non target organisms. Organisms exposed to environmental concentrations of acetaminophen (AMP), diclofenac (DIC), ibuprofen (IBU), ketoprofen (KET) and nimesulide (NIM) revealed a significant accumulation of DIC, IBU and NIM, while AMP and KET were always below detection limit. Nonetheless, for all tested NSAIDs, measurement of a large panel of ecotoxicological biomarkers highlighted impairment of immunological parameters, onset of genotoxicity and modulation of lipid metabolism, oxidative and neurotoxic effects. Laboratory results were integrated with a field study which provided the first evidence on the occurrence of DIC, IBU and NIM in tissues of wild mussels sampled during summer months from an unpolluted, touristic area of Central Adriatic Sea. Overall results demonstrated *M. galloprovincialis* as a good sentinel species for monitoring presence and ecotoxicological hazard of pharmaceuticals in the Mediterranean.

Keywords: Emerging contaminants, Pharmaceuticals, Non Steroidal Anti-Inflammatory Drugs, *Mytilus galloprovincialis*, bioaccumulation, biomarkers, Adriatic Sea.

1. Introduction

The presence of pharmaceutical compounds in aquatic ecosystems has became a topic of growing concern in the last decade (Fent *et al.,* 2006; Boxall *et al.,* 2012). The occurrence of such emerging contaminants in the aquatic environment originates from the large consumption in human and veterinary medicine, agriculture and aquaculture (Boxall *et al.,* 2012) and the limited removal of these molecules by many wastewater treatment plants (WWTPs, Santos *et al., 2010*).

Compounds used as contraceptives, beta-blockers, antiepileptic, anti-inflammatory, antidepressants or antibiotics have been documented at concentrations ranging from a few ng/L to µg/L in surface and ground waters around the world (Santos *et al.,* 2010; Al Aukidy *et al.,* 2012). Designed to be biologically active at low concentrations, environmental pharmaceuticals might be potentially dangerous for chronically exposed, non-target organisms (Fent *et al.,* 2006; Boxall *et al.,* 2012). This new awareness was reflected in some international actions: the European Medicine Agency (EMEA) issued in 2006 the Guideline on Environmental Risk Assessment Of Medicinal Products for Human Use, aimed to evaluate the risks of pharmaceuticals in ecosystems; the European Parliament extended in September 2010 the legislation on pharmaco-vigilance (Directive 2001/83 and Regulation 726/2004) to the environment (ecopharmacovigilance); more recently, European Commission added 17α-ethinyl estradiol, 17β-estradiol and diclofenac to the Watch List of the daughter Water Framework Directive (2013/39/EU). Overall, the urgent need is recognized for prioritizing more than 4000 substances in terms of their occurrence in natural ecosystems, bioavailability for non target aquatic organisms, mechanisms of uptake and biotransformation, mode of action at subcellular level, onset of biologically adverse effects and ecotoxicological relevance (Boxall *et al.,* 2012).

Non steroidal anti-inflammatory drugs (NSAIDs) are one of the most relevant therapeutic class, largely used worldwide for their analgesic, antipyretic and anti-inflammatory properties. They act as non-selective inhibitors of cyclooxygenase isoforms, COX-1 and COX-2, involved in the synthesis of different prostaglandins from arachidonic acid (Santos *et al.,* 2010). Their annual consumption in Italy is estimated higher than 100 ton (OsMed, 2013), and they represent 15% of drugs detected in monitoring surveys worldwide (Santos *et al.,* 2010). Despite NSAIDs residues are ubiquitously present in surface waters, groundwaters and coastal areas (Pal *et al.,* 2010; Santos *et al.,* 2010; Lolić *et al.,* 2015), our knowledge on their bioaccumulation and ecotoxicological potential is still limited, particularly for marine organisms. In this context, bivalves are universally considered as useful bioindicator species due to their worldwide distribution, sedentary and filter-feeding habit, easy sampling, elevated tolerance to environmental conditions and marked capability to accumulate organic and inorganic chemicals. Moreover, the possibility to measure several

biochemical, cellular and physiological biomarkers, make bivalves suitable organisms for investigating the effects of chemical pollutants (Regoli et al., 2014)

Haemocytes of the freshwater mussels *Dreissena polymorpha* revealed DNA fragmentation and enhanced percentage of apoptotic cells when exposed to various doses of acetaminophen (30-450 µg/L), diclofenac (60-250 µg/L) and ibuprofen (450-909 µg/L) (Parolini *et al.,* 2009). More limited variations of the same parameters were reported after *in vivo* exposure of *D. polymorpha* to lower concentrations of acetaminophen (0.75-1.51 µg/L) and ibuprofen (2-8 µg/L), along with modulation of antioxidant enzymes (catalase, glutathione peroxidases and glutathione S-transferases) and a decrement of lysosomal membrane stability (Parolini *et al.,* 2010, 2011).

The marine clam, *Ruditapes philippinarum*, exhibited significant immunological alterations after 7 days of exposure to ibuprofen (500 and 1000 µg/L) (Matozzo *et al.,* 2012). The same species, exposed for 7 days to 100 µg/L ibuprofen, revealed transcriptional changes for several genes involved in arachidonic acid metabolisms, apoptosis, peroxisomal proliferator-activated receptors, nuclear factor-kappa B and xenobiotic metabolisms (Milan *et al.*, 2013); these effects were paralleled by a statistically significant inhibition of superoxide dismutase, acetylcholinesterase and lysozyme activities (Milan *et al.,* 2013). Byssus strength and energy available for growth and reproduction decreased in *Mytilus edulis* exposed to 100 and 1000 µg/L of diclofenac for 7 and 14 days (Ericson *et al.,* 2010). In the Mediterranean mussel, *M. galloprovincialis*, ibuprofen and diclofenac (250 ng/L) determined, after two weeks, a transitory induction of antioxidant enzymes (superoxide dismutase, catalase, glutathione S-transferases and glutathione reductase) and increase of lipid peroxidation in digestive gland; ibuprofen also enhanced the levels of gonad vitellogenin-like proteins in males, suggesting a potential role as endocrine disruptor of this molecule for marine mussels (Gonzalez-Rey and Bebianno 2011, 2012, 2014).

The aim of this study was to provide new insights on ecotoxicity of pharmaceuticals by comparing the bioaccumulation and the responsiveness of *M. galloprovincialis* toward 5 different NSAIDs with similar characteristics in terms of mode of action, posology and therapeutic indication for human beings. Mussels were exposed to Acetaminophen (AMP), Diclofenac (DIC), Ibuprofen (IBU), Ketoprofen (KET) or Nimesulide (NIM), and chemical analyses on bioaccumulation of such NSAIDs in mussel tissues were integrated with a multi-biomarker approach, based on a wide array of molecular and subcellular responses reflecting early warning signals of biological disturbance, modulation of specific cellular pathways, onset of various typologies of cellular damages and toxicity. Selected biomarkers included lysosomal and immunological responses (Neutral red retention time NRRT, granulocytes-hyalinocytes ratio, phagocytosis capacity), lipid peroxidation (lipofuscin and neutral lipids), peroxisomal proliferation (Acyl CoA oxidase ACOX),

neurotransmission system (AChE), levels of antioxidant defenses (CAT, GST, GR, GPx, and levels of glutathione GSH), total oxyradical scavenging capacity (TOSC) and genotoxic effects (DNA integrity and micronuclei MN). Results on biomarker responses were elaborated within a recently developed, quantitative model (Sediqualsoft) which integrates and differently weight large data-sets of biomarker variations, providing synthetic indices of hazard based on number, typology, biological relevance and magnitude of observed effects (Piva *et al.,* 2011; Benedetti *et al.,* 2012; Regoli *et al.,* 2014).

Results on bioaccumulation under laboratory conditions were also compared with values detected for the first time in a wild mussels population sampled during summer months from an Adriatic touristic location.

Obtained results were expected to clarify the potential role of *M. galloprovincialis* as suitable sentinel organism to evaluate bioavailability and potentially adverse effects of NSAIDs in marine ecosystems. The comparison between different NSAIDs could also reveal whether molecules belonging to the same therapeutic class could cause different effects on non target organisms, thus providing useful insights for a preliminary prioritization on ecological sustainability among tested molecules.

2. Materials and Methods

2.1 Chemicals

Acetaminophen, AMP (CAS 103-90-2), Diclofenac, DIC (CAS 15307-86-5), Ibuprofen, IBU (CAS 15687-27-1), Ketoprofen, KET (CAS 22071-15-4) and Nimesulide, NIM (CAS 51803-78-2) were obtained from Sigma Aldrich (Milan, Italy); these chemicals were used for both the exposure treatments and for analytical purposes.

2.2 Mussel exposure

Mussels *M. galloprovincialis* (5 ± 1 cm shell length) were obtained from a local farm (Ancona, Adriatic Sea); 360 mussels were randomly distributed into six 20 L aquarium (60 mussels per tank) and acclimatized for 10 days to laboratory conditions with aerated seawater, at 18 ± 1°C, salinity 37, pH 8.0 ± 0.5 and oxygen saturation >94%. Due to their low solubility in water, stock solutions of AMP, DIC, IBU, KET and NIM were prepared in methanol and stored at room temperature for the duration of the experiment. Working solutions were prepared daily by diluting the stock solutions in seawater.

The experimental design included five tanks with organisms exposed to 25 µg/L of AMP, DIC, IBU, KET and NIM respectively and a solvent control tank (CTRL) where methanol was added at the same concentration used in the NSAIDs treatments (0.003%). The chosen exposure concentration is higher than those typically found in marine field conditions (from a few units to hundreds of ng/L; Gros et al., 2012; Lolić *et al.,* 2015), but still environmentally realistic for particularly challenged sites (up to tens of µg/L; Togola and Budzinski 2008). Water was changed daily, redosing concentrations of molecules and solvent; no mortality was observed during the experiment. After 14 days, 30 specimens for each treatment were dissected for chemical analyses, whole tissues were pooled in 5 samples (each containing tissues of 6 organisms) and stored at −20°C. The remaining 30 specimens for each treatment were used for biological analyses preparing 10 replicates of haemolymph, digestive glands and gills, each constituted by tissues of 3 specimens. Gills and digestive glands were frozen in liquid nitrogen and maintained at -80°C for the biomarker analyses. Small pieces of digestive glands were also excised from the 10 specimens of each group and frozen in hexane precooled to -70°C in liquid nitrogen, for histochemical analyses. Haemolymph was withdrawn from the adductor muscle and divided in three aliquots: the first was frozen in liquid nitrogen and maintained at -80 °C for the acetylcholinesterase analysis; another aliquot was immediately processed for the analyses of neutral red retention time (NRRT), granulocytes-hyalinocytes ratio, phagocytosis activity and DNA damage; the last aliquot of haemolymph was fixed in Carnoy's solution (3:1 methanol, acetic acid) for the microscopic evaluation of micronuclei frequency.

2.3 Field investigation

Wild mussels, *M. galloprovincialis* (5.0 ± 1.0 cm shell length), were sampled during the summer period (July, August and September 2014) in the Portonovo Bay, located in the Central Adriatic sea. This is an enclosed area considered as a pristine site and representing an important touristic destination. After collection, whole tissues were removed from 30 specimens, pooled in 10 samples (each containing 3 organisms), and stored at −20°C until analysed for NSAIDs bioaccumulation.

2.4 Chemical analyses

Bioaccumulation of AMP, DIC, IBU, KET and NIM in mussels was determined on 5 replicates, each constituted by whole tissues of 6 specimens. Different homogenization and extraction buffers were used for various compounds: acetic acid 0.1%, pH = 3.26 (buffer 1) for DIC, IBU, KET and NIM, while ammonium phosphate 10 mM, pH = 4.0 with citric acid 100 mM (buffer 2) for AMP. About 3 g of wet tissues were homogenized in 5mL of buffer at room temperature for 20 minutes.

After centrifugation at 4500 ×g for 30 minutes, samples were purified by Solid Phase Extraction (SPE) with reversed-phase tubes (Discovery DSC-18, 1g x 6 mL, Supelco, Bellefonte, Pennsylvania, USA). SPE tubes were conditioned with 6 mL of methanol, followed by 18 mL of ultra-pure water, and by 12 mL of buffer. Samples were loaded onto the SPE cartridges, and after washing with 12 mL of ultra-pure water, analytes were eluted and recovered using 3 mL of acetonitrile (HPLC, gradient grade, Carlo Erba). Obtained samples were filtered using Phenex™-RC membrane (Regenerated Cellulose/Polypropylene 0.45 µm, 15mm syringe filters, Phenomenex, US) and then concentrated by SpeedVac at room temperature to a final volume of 1 mL. Concentrated samples were centrifuged again at 12000 ×g for 20 minutes. Analytical detection of extracted NSAIDs was performed by High Performance Liquid Chromatography, with fluorimetric and diode array detectors DAD (Agilent Infinity 1260 series). Chromatographic separations of DIC, IBU, KET and NIM were performed on a Kinetex column (C18, 5 µm, 150 mm length, 4.6 mmID, Phenomenex, US), equipped with a security guard column (C18, 5 µm, 4 mm length, 2.0 mmID, Phenomenex, US). For KET and DIC a mobile phase composed by ultra-pure water (26%), acetonitrile (42%) and Buffer 1 (32%) was used under isocratic condition. DAD was used for monitoring the spectra from 190 nm to 350 nm, and the signal for KET and DIC was obtained at 250 nm and 276 nm respectively. These wavelengths were selected among the spectra ranges assuring at least the 85% of the maximum absorbance, verifying the absence of other spectral interferences; additional qualifying signals (with about 50%-75% of the maximum absorbance) were also recorded for each compound for the quality control and assurance. When necessary, the full spectra was used to verify the purity of the obtained peaks, comparing them with those obtained from pure standard solutions. Analyses of IBU and NIM were performed using ultra-pure water, acetonitrile, Buffer 1 gradient (from 35%:30%:35% to 0%:65%:35% linearly during 23 minutes). Analytical measurement of IBU was obtained by fluorimetric detector with excitation/emission wavelengths at 230/294 nm, while NIM was detected using DAD from 190 nm to 410 nm and monitoring at 298 nm. Also for this molecule, additional qualifying signals were obtained for quality control and assurance and pure standard solutions were used to further verify the purity of the obtained peaks. Separation of AMP was carried out by an Agilent Eclipse Plus column (C18, 3.5 µm, 100 mm length, 4.6 mmID) with a security guard column (C18, 5 µm, 4 mm length, 2.0 mmID, Phenomenex, US), and a mobile phase composed by Buffer 2 (87.5%) and methanol (12.5%) under isocratic condition; a fast, post-run gradient was applied to remove any unresolved compound retained by the analytical column (from 87.5%:12.5% to 10%:90%). Detection was obtained using diode array in the range 190-350 nm, monitoring the signal at 248 nm and checking for quality control and assurance with additional qualifying signals. Concentrations of various

NSAIDs were quantified by comparison with signals of pure standard solutions. Due to the lack of appropriate Certified Standard Reference Materials (SRMs), recovery for each compound was estimated on samples of control mussels (n=10) spiked with various concentrations of investigated molecules. The minimum value of the optimal working range corresponded to the analytical limit of measurement which guarantees an acceptable variability (CV<20%) on 10 replicates and a good linearity ($R^2 \geq 0.99$), while the maximum value assured at least 95% of recovery (n=10). These ranges were respectively 0.39-1000 ng/mL for AMP, 0.62-1000 ng/mL for DIC, 0.29-1000 ng/mL for IBU, 0.27-1000 ng/mL for KET and 0.45-1000 ng/mL for NIM; considering these analytical conditions and the described preparation procedures, the minimum measurable amounts (Limit of Detection, LOD) in mussels tissues were fixed to 1 ng/g dry weight (d.w.) for AMP, DIC and NIM, and 0.5 ng/g (d.w.) for IBU and KET. All those values always ensure an appropriate analytical accuracy. During the protocols validation, samples spiked with levels of NSAIDs in the range of our experimental design, always provided significant reproducibility of results with low variability (CV<5%, n=10) and elevated recovery yield (≥98%).

2.5 Biomarker analyses

All the procedures for biomarker analyses followed standardized protocols (Gorbi et al., 2013) which have been fully detailed in Supplementary Material 1 (SM1). Briefly, analyzed parameters included: immunological alterations in haemocytes (in terms of lysosomal membrane stability (NRRT), granolocytes/hyalinocytes ratio and phagocytosis activity), lipofuscin and neutral lipids accumulation in the digestive glands, peroxisomal proliferation index (acyl-CoA oxidase activity (ACOX)), neurotoxic responses as acetylcholinesterase (AChE) in haemocytes and gills, oxidative stress biomarkers in digestive tissues (i.e. catalase, glutathione S-transferases, glutathione peroxidases and glutathione reductase activities and total glutathione), total oxyradical scavenging capacity (TOSC), genotoxic effects in haemolymph in terms of DNA strand breaks and micronuclei frequency (MN).

2.6 Statistical analyses

Analysis of variance (ANOVA) was applied for all the analyzed parameters to test differences between the experimental conditions. Level of significance was set at $p < 0.05$, homogeneity of variance was checked by Cochram C and mathematical transformation applied if necessary; post-hoc comparison (Newman-Keuls) was used to discriminate between means of values (n=5). Descriptive multivariate statistical analysis (PCA, principal component analyses) was carried out to discriminate between different exposure conditions. Results on biomarker responses

in mussels exposed to NSAIDs compounds were further elaborated within a quantitative Weight Of Evidence, WOE model (Sediqualsoft) which integrate large data-sets of heterogeneous data, or lines of evidence (LOEs) including sediment chemistry, bioaccumulation of chemicals in key sentinel species, sublethal effects measured through biomarkers and toxicological effects at organisms level assessed by standardized laboratory bioassays. Each LOEs are independently elaborated before their differential weighting and integration in a quantitative WOE evaluation which provide a synthetic index of risk. As results huge amount of complexes data from various LOEs can be summarized into indices that maintain scientifically sound information, while also being easy to read for non-expert stakeholders (Piva *et al.,* 2011; Benedetti *et al.,* 2012; Regoli *et al.,* 2014). For elaboration of biomarker results, each parameter has a specific weight (based on toxicological relevance of measured endpoint) and a threshold that indicates the minimum percentage variation considered of biological relevance for that biomarker response (Piva *et al.,* 2011;). For every analysed biomarker, the measured variation is compared to the threshold, then corrected for the weight of the response and the statistical significance of the difference compared to controls. The calculation of the Hazard Quotient for biomarkers (HQ_{BM}) does not consider the contribution of responses with an effect lower or equal to threshold, calculates the average for those with an effect up to two-fold compared to the threshold and adds the summation (Σ) for the responses more than 2 fold greater than the respective threshold (Piva et al., 2011):

$$HQ_{BM} = \left(\frac{\sum_{j=1}^{N} Effect_W(j)_{1<Effect(j)\leq 2}}{num\ biomark_{1<Effect(j)\leq 2}} + \sum_{k=1}^{M} Effect_W(k)_{Effect(j)>2} \right)$$

According to variations measured for various biomarkers, the model summarizes the level of cumulative HQ_{BM} in one of five classes of hazard for biomarkers, from Absent to Severe. The rationale, mathematical algorithms and logical flow-charts have been fully detailed elsewhere (Piva *et al.,* 2011).

3. Results

Mussels exposed to various NSAIDs showed a significant bioaccumulation of DIC, IBU and NIM, with tissue concentrations increasing from below detection limits up to 14.9 ± 7.89 ng/g, 1.63 ± 1.00 ng/g, and 30.22 ± 13.50 ng/g d.w., respectively (Table 1). On the other hand, no variations were observed after 14 days for AMP and KET in mussels tissues, remaining below the limit of 1 ng/g and 0.5 ng/g d.w., respectively (Table 1). Chemical analyses on wild mussels collected in the coastal area of Portonovo confirmed the lack of detectable accumulation of AMP and KET over the whole sampling period (Table 1). On the other hand, levels of DIC and IBU were below detection limit in July and September, while concentrations measured in August were comparable to those obtained after laboratory exposures for DIC and almost 6 folds higher for IBU (Table 1). NIM was always detected in wild organisms, with tissue concentrations rather comparable in different sampling months and lower compared to those obtained after laboratory exposures (Table 1).

The analysis of biomarker responses in laboratory exposed mussels revealed that, among immunological parameters, lysosomal membrane stability was significantly reduced by all NSAIDs with more elevated effects in organisms exposed to DIC, IBU and NIM (Fig. 1A). Granulocytes-hyalinocytes ratio and phagocytosis activity did not exhibit any variation in exposed mussels (Fig. 1B-C). A significant accumulation of lipofuscin in tertiary lysosomes occurred in all treatments, particularly in DIC and IBU exposed organisms (Fig. 1D), while only a limited, not significant decreasing trend was observed for neutral lipids (Fig. 1E). A marked inhibition of the acyl CoA oxidase activity was observed in organisms exposed to all the tested NSAIDs (Fig. 1F); the activity of acetylcholinesterase was significantly modulated only in haemolymph of organisms exposed to AMP, with no effect by other molecules and in gills (Fig. 1 G-H).

Among antioxidant defences, catalase activity showed a significant inhibition after exposure to KET and NIM (Fig. 2A), while IBU caused the decrease of glutathione S- transferases (Fig. 2B). The exposure to NSAIDs did not cause any change in the activities of glutathione peroxidases (both Se-dependent and the sum of Se-dependent and Se-independent forms), glutathione reductase, and levels of total glutathione (Fig. 2C-F). The limited prooxidant effects induced by NSAIDs on exposed mussels were further supported by the analyses of the Total Oxyradical Scavenging Capacity, with a statistically significant decrease of TOSC only toward •OH, for organisms exposed to NIM (Fig. 2 G-H).

Genotoxic effects were revealed by the enhancement of DNA strand breaks particularly in IBU and DIC exposed organisms (Fig. 3A), and the marked increase of micronuclei frequency in mussels exposed to all NSAIDs (Fig. 3B).

The principal component analysis (PCA), carried out on the whole set of biomarker responses, produced a two dimensional pattern explaining 69.39% of the total variance, with a clear separation between the control (CTRL) and all exposed organisms (AMP, DIC, IBU, KET and NIM) (Fig. 4). The parameters determining the separation along to the PC1 axis were those related to cellular damage (lysosomal membrane stability, accumulation of lipofuscin and neutral lipids, micronuclei frequency and DNA strand breaks), while granulocytes-halynocites ratio, acetylcholinesterase in hemolymph and total oxyradical scavenging capacity toward peroxyl radicals, determined the separation of the groups along to the PC2 axis. Considering the biological relevance (weight) and magnitude of observed effects, the elaboration of biomarker results within the Weight of Evidence model summarized as "Moderate" the hazard for organisms exposed to AMP DIC, IBU, KET and NIM (Table 2).

4. Discussion

Over the last fifteen years, pharmaceuticals have emerged as an important class of environmental contaminants, with a documented occurrence in freshwater ecosystems, and potential adverse effects on non target organisms (Parolini *et al.,* 2009; Santos *et al.,* 2010, Al Aukidy *et al.,* 2012). Relatively less attention has been paid to the ecotoxicological consequences of pharmaceuticals released in the marine environment, despite recent investigations reported the presence of about 113 pharmaceutical compounds in worldwide coastal waters at concentrations ranging from 0.01 to 6800 ng/L (Gaw *et al.,* 2014).

To improve our knowledge and awareness on this issue, the present study was aimed to evaluate the sensitivity of a typical marine bioindicator, the filter feeding *Mytilus galloprovincialis*, toward various pharmaceutical molecules. Novel aspects of the research included an integrated ecotoxicological approach combining bioaccumulation data with a wide spectrum of subcellular responses, the comparison of different molecules of the same and widely used therapeutic class (NSAIDs), the first assessment of the natural occurrence of NSAIDs in wild mussels sampled from a touristic coastal area in the Adriatic Sea.

Although various analytical methods have been reported for the determination of pharmaceuticals in environmental abiotic matrices (Al Aukidy *et al.,* 2012; Gros *et al.,* 2012) more limited protocols are available for their extraction and analysis in aquatic organisms (Huerta et al. 2013; Miller *et al.,* 2015). The successful development of specific HPLC procedures to quantify NSAIDs concentration in mussels tissues allowed to characterize the capability of *M. galloprovincialis* to accumulate the tested pharmaceuticals.

Results obtained from laboratory exposures, indicated NIM as the most accumulated drug in mussels, followed by DIC and IBU. No information was previously available on the capability of Mediterranean mussels to concentrate NSAIDs in their tissues, and only few data had been reported for aquatic organisms. NIM has been recently detected in freshwater amphipod *Gammarus pulex* from the Thames River at values from below detection limit to 16 ng/g d.w. (Miller *et al.,* 2015), while DIC concentrations of 4.1 and 8.8 ng/g d.w were measured the wild fish *Barbus graellsii* and *Micropterus salmoides,* respectively, from Spanish rivers (Huerta et al. 2013). Under laboratory conditions, the fathead minnow *Pimephales promelas* exposed for 28 days to 250 µg/L of IBU exhibited tissue levels of 104, 167 and 105 ng/g w.w. in muscle, gills and liver respectively (Nallani *et al.,* 2011), while in zebra mussel *D. polymorfa* concentrations raised to 398.9 ± 59.8 ng/g d.w. after 7 days at 20.6 µg/L (Contardo-Jara *et al.,* 2011). No information is actually available on accumulation of AMP and KET in aquatic organisms and, based on our results, no detectable levels of these molecules were observed in exposed *M. galloprovincialis*. While a limited bioavailability of AMP could be hypothesized due to the low octanol-water partition coefficient (log Kow 0.46), KET has a log Kow (3.12) similar to those of the other investigated and accumulated NSAIDs. In addition, the lack of AMP and KET accumulation in mussels tissues does not exclude an ecotoxicological concern for these molecules since the original compounds might be transformed either in the environment or within the organisms. The hypothesis of a biological reactivity of NSAIDs in non target marine organisms even in the absence of elevated bioconcentration is supported by the early onset of molecular and cellular responses evaluated through a wide battery of validated biomarkers.

One of the main evidence of this work was the onset of cytotoxic effects in haemocytes with a significant decrement of lysosomal membrane stability for all the treatments. In bivalves haemocytes are primarily involved in cell physiology, intracellular turnover, immune responses, degradation and eliminations of pathogens (Gorbi et al.,2013): obtained results confirmed the elevated sensitivity of these responses for detecting adverse effects of anti-inflammatory compounds in non target marine organisms.

Alterations of lysosomal membrane stability were previously reported for *D. polymorfa* exposed to AMP, DIC and IBU (Parolini *et al.,* 2009, 2010, 2011), and in *R. philippinarum* exposed to 10 and 50 µg/L of IBU (Aguirre Matinez *et al.,* 2013). The impairment of haemocytes lysosomal stability would confirm the modulation of immune parameters by the COX-based mode of action elicited by NSAIDs also in aquatic invertebrates (Gonzalez-Rey and Bebianno, 2012; Aguirre Matinez *et al.,*2013, Milan *et al.,* 2013).

Despite the decrease of NRRT, no significant effects were observed for phagocytosis, and the limited variation observed for granulocytes-hyalinocytes ratio may represent an early phase response due to modulation of cellular turnover by NSAIDs.

Histological analyses revealed a marked accumulation of lipofuscin in digestive gland and a decreasing content of neutral lipids in all treated organisms. Lipofuscin accumulation is considered a marker of autophagic processes in Mediterranean mussels and indicative of peroxidative processes, while neutral lipids represent one of the major energy reserves in bivalves (Bocchetti *et al.,* 2008). The reduction of neutral lipid content can be related to the inhibition of acyl-CoA oxidase activity, an effect observed for all tested NSAIDs. Peroxisomal enzymes modulate key pathways of lipid metabolism, such as β-oxidation of very long-chain fatty acids and important lipid derivatives like prostaglandins and leukotrienes, synthesis of plasmalogens and cholesterol, which acts as precursor of steroid hormones (Cajaraville and Ortiz-Zarragoitia, 2006). Considering our results, it can be suggested that NSAIDs reduce β-oxidation pathways and determine an increased consumption of energy reserves in mussels. Despite the knowledge on modulation of lipid metabolism by NSAIDs is still limited in invertebrates, inhibitory effects on fatty acid oxidation have been reported in isolated rat liver mitochondria and hepatocytes (Zhao *et al.,* 1992); additional lipid-modulating actions of NSAIDs have been recently studied to counteract atherosclerosis in humans (Diapen *et al.,* 2013).

Acetylcholinesterase has a key role in terminating neurotransmission at cholinergic synapse and its modulation in haemolymph and gills of bivalves has been demonstrated for a wide spectrum of environmental pollutants (Galloway *et al.,* 2002; Gorbi *et al.,* 2008; Regoli *et al.,* 2014; Rickwood and Galloway, 2004; Solè *et al.,* 2010). The sensitivity of AchE activity to emerging compounds such as NSAIDs is currently poorly documented in *M. galloprovincialis*; in our study, this biomarker showed limited variations with a slight enhancement in haemolymph of AMP and NIM-exposed mussels and no further changes or a trend toward decreased values in gills for most of the treatments. To our knowledge, no information was available on AchE modulation on hemolymph in mussels challenged by NSAIDs, but previous studies demonstrated the effects of NSAIDs on AchE in gills of mussels *M. galloprovincialis*, with induction caused by DIC (Gonzalez-Rey and Bebianno, 2014), and inhibition by AMP (Solè *et al.,* 2010). Our results support the hypothesis that also NSAIDs may influence the activity of this enzyme, with tissue-specific responses, but mechanisms of action still remain unclear.

Prooxidant mechanisms are of primary importance in modulating toxicological effects of xenobiotics in marine organisms, and the measurement of oxidative stress biomarkers is useful from the early detection of a general disturbance to later impairment of organisms health condition

(Gorbi *et al.,* 2013). In this study, the oxidative status of exposed mussels was assessed by integrating the measurement of individual antioxidants with the total capability to neutralize specific oxyradicals. Slightly lowered values were measured for catalase, glutathione S-transferases and TOSC toward hydroxyl radicals, in agreement with a previously reported inhibition of catalase in mussels exposed to DIC and IBU (Gonzalez-Rey and Bebianno, 2012, 2014). Considering the specific functions of these systems in preventing the formation of hydroxyl radicals from hydrogen peroxide, removing oxidative damages and limiting membrane lipid peroxidation, these variations might contribute to explain the previously described increase in lipofuscin content (Regoli and Giuliani, 2014). The lack of changes observed for all the other antioxidants and the peroxyl radical scavenging capacity corroborate the occurrence of an overall limited oxidative pressure caused by NSAIDs, as already shown in mussels (Gonzalez-Rey and Bebianno, 2011, 2012).

Despite pharmacodynamics studies exclude genotoxic effects of NSAIDs in mammals, significantly higher levels of DNA strand breaks and micronuclei frequency were observed in all exposed mussels, confirming similar effects reported in *D. polymorpha* after *in vitro* and *in vivo* exposures to ibuprofen (Parolini *et al.,* 2009, 2011). The parallel onset of DNA fragmentation with loss of lysosomal stability and accumulation of lipofuscin, further support the hypothesis of a common oxidative mechanism of cell damage. On the other hand, the enhanced frequency of micronuclei might be partly related to changes in cell cycle caused by NSAIDs, rather than direct genotoxic effects, as already shown in mammalian models (Chang *et al.,* 2009).

The multivariate PCA analysis of biomarker results provided a clear separation between control mussels and those exposed to various NSAIDs indicating that onset of biological effects was not necessarily related to the bioaccumulation of tested pharmaceuticals. In this respect, when biomarker variations were elaborated through weighted criteria which consider number, magnitude and biological relevance of observed effects, the level of hazard in mussels exposed to NSAIDs was summarized as Moderate, confirming the utility of summarizing complex biological results in an easy index to enhance the visibility of ecotoxicological concern for environmental pharmaceuticals also in non toxicologists.

Field results provided the first evidence on the occurrence of NSAIDs in tissues of wild mussels sampled from an unpolluted coastal area of Adriatic Sea. Concentrations of NIM were always revealed during the summer season 2014, while those of DIC and IBU were detectable only in August. The observed monthly variations might, at least partially be related to meteorological conditions of summer 2014 when average rain precipitations in July and September were 2-3 folds higher than the typical seasonal values and with some particularly marked events which determined a limited touristic presence at this site (see http://www.meteo.marche.it/news/estate2014.pdf, in

italian). In this respect, the relatively constant levels of NIM in mussels, despite lower than those measured in laboratory conditions, might suggest a certain environmental persistence of this molecule. Concentrations of DIC and IBU measured only in August in wild mussels were comparable or even higher than those obtained in laboratory conditions, despite the probably lower environmental exposure dose which further corroborate the sensitivity of mussels as bioindicators for these pharmaceuticals. Preliminary laboratory exposures demonstrated the capability of mussels to completely excrete DIC, IBU or NIM after 30 days of depuration (data not shown), suggesting that pharmaceuticals measured in wild organisms reflected an actual bioavailability of these molecules in the environment. Levels of AMP and KET were not detectable in field organisms but, according to experimental results, the lack of accumulation might be due to transformation or metabolization of these pharmaceuticals.

In conclusion, this study demonstrated *M. galloprovincialis* as a good sentinel species for monitoring presence and ecotoxicological hazard of pharmaceuticals in the Mediterranean. The comparison between laboratory studies and field investigation, highlighted similar trends of drug accumulation, with differences among various NSAIDs: AMP and KET were never detectable, while DIC, IBU and NIM were easily measured in mussels. However, even in the absence of enhanced tissues concentrations, clear biological effects revealed modulation of specific biochemical pathways and onset of various forms of cellular damage. Further studies are needed on the ecotoxicological potential of NSAIDs, particularly to fill our knowledge gaps on occurrence and biological effects of these pollutants in field conditions, considering larger spatial and temporal scales, as also required by Marine Strategy Framework Directive.

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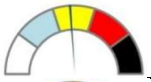
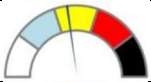
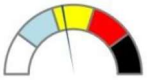
TABLES

Table 1. Bioaccumulation of acetaminophen (AMP), diclofenac (DIC), ibuprofen (IBU), ketoprofen (KET) and nimesulide (NIM) in mussels, *M. galloprovincialis*, exposed in laboratory conditions and in those sampled in field conditions. Data are given as ng/g d.w. (mean values ± standard deviation, n=5).

		AMP	DIC	IBU	KET	NIM
Exposure	Control	<LOD	<LOD	<LOD	<LOD	<LOD
	Exposed	<LOD	14,90 ± 7,89	1,63 ± 1,00	<LOD	30,22 ± 13,50
Field Investigation	Jul-2014	<LOD	<LOD	<LOD	<LOD	6,04 ± 10,47
	Aug-2014	<LOD	16,11 ± 14,72	9,39 ± 0,59	<LOD	4,18 ± 2,54
	Sept-2014	<LOD	<LOD	<LOD	<LOD	2,99 ± 5,18

LOD: limit of detection

Table 2. Quantitative Hazard Quotients (HQ) and assigned Class of hazard after weighted elaboration of biomarkers data in mussels exposed to different NSAIDs.

Treatment	HQ	Class of hazard	Level
AMP	19,78	MODERATE	
DIC	27,40	MODERATE	
IBU	23,95	MODERATE	
KET	17,99	MODERATE	
NIM	20,51	MODERATE	

LEGENDS OF FIGURES

Figure 1. Lysosomal stability, granulocytes-hyalinocytes ratio, phagocytosis, lipofuscin, neutral lipids, Acyl-CoA oxidase (ACOX) and acetylcholinesterase activities in mussels exposed to acetaminophen (AMP), diclofenac (DIC), ibuprofen (IBU), ketoprofen (KET) and nimesulide (NIM) for 14 days (mean values ± standard deviations, n=5). Different letters indicate significant differences between groups of means.

Fig**ure** 2. Antioxidant defenses and total oxyradical scavenging capacity (TOSC) toward peroxyl (ROO•) and hydroxyl (HO•) radicals in mussels exposed to acetaminophen (AMP), diclofenac (DIC), ibuprofen (IBU), ketoprofen (KET) and nimesulide (NIM) for 14 days (mean values ± standard deviations, n=5). Different letters indicate significant differences between groups of means.

Figure 3. Biomarkers of genotoxic damage: DNA integrity and micronuclei frequency measured in mussels exposed to acetaminophen (AMP), diclofenac (DIC), ibuprofen (IBU), ketoprofen (KET) and nimesulide (NIM) for 14 days (mean values ± standard deviations, n=5). Different letters indicate significant differences between groups of means.

Figure 4. PCA analysis on biomarker data in control mussels (CTRL) and in those exposed to acetaminophen (AMP), diclofenac (DIC), ibuprofen (IBU), ketoprofen (KET) and nimesulide (NIM). G/H = granulocytes-hyalinocytes ratio; AchE= acetylcholinesterase in haemolymph; TOSC ROO•= Total Oxyradical Scavenging Capacity toward peroxyl radicals; NRRT= neutral red retention time; LIPO= lipofuscin; ORO= neutral lipids; DNA sb= DNA strand breaks; MN= micronuclei frequency; GPx H2O2= Se-dependent glutathione peroxidases

Figures

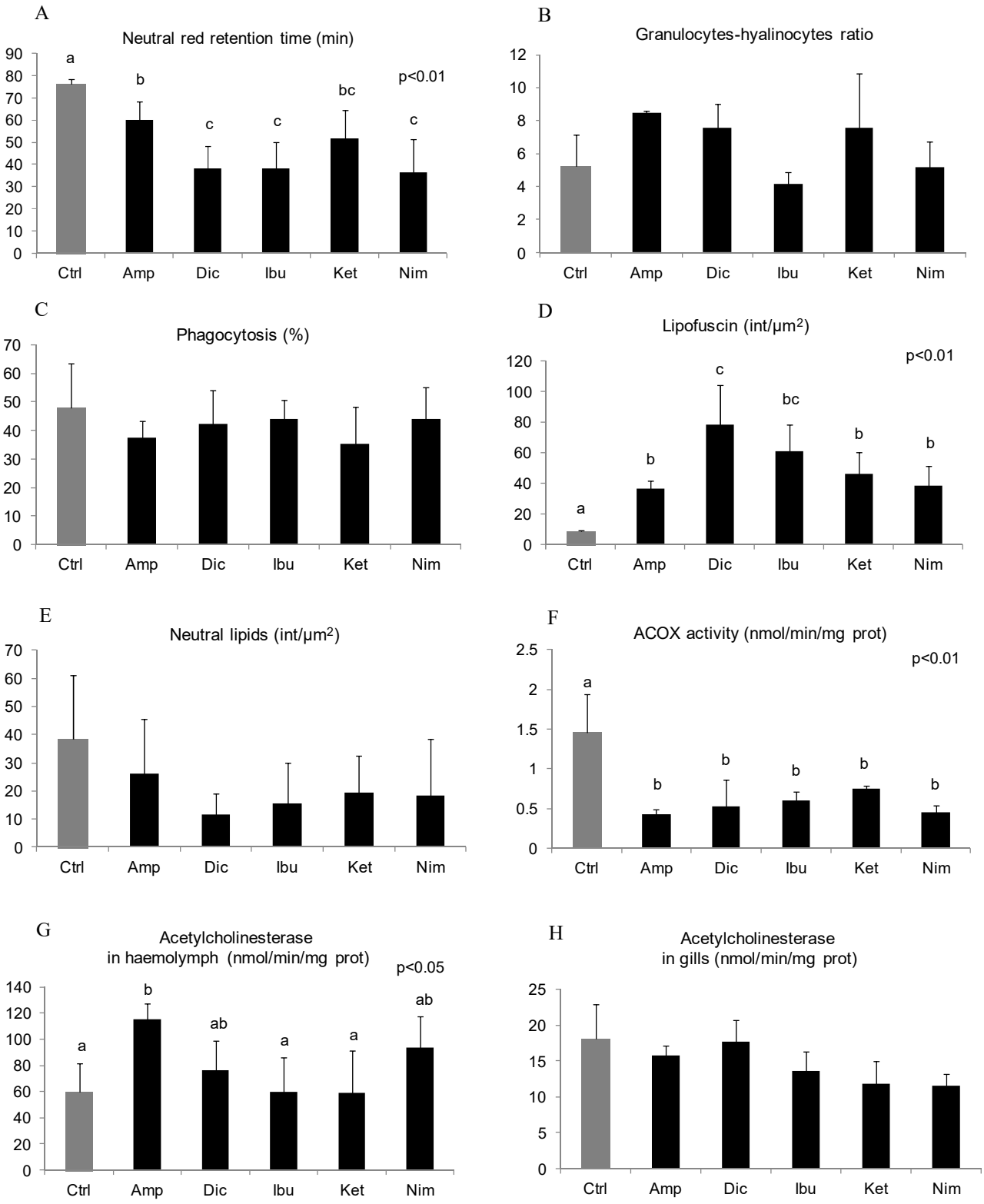


Figure 1.

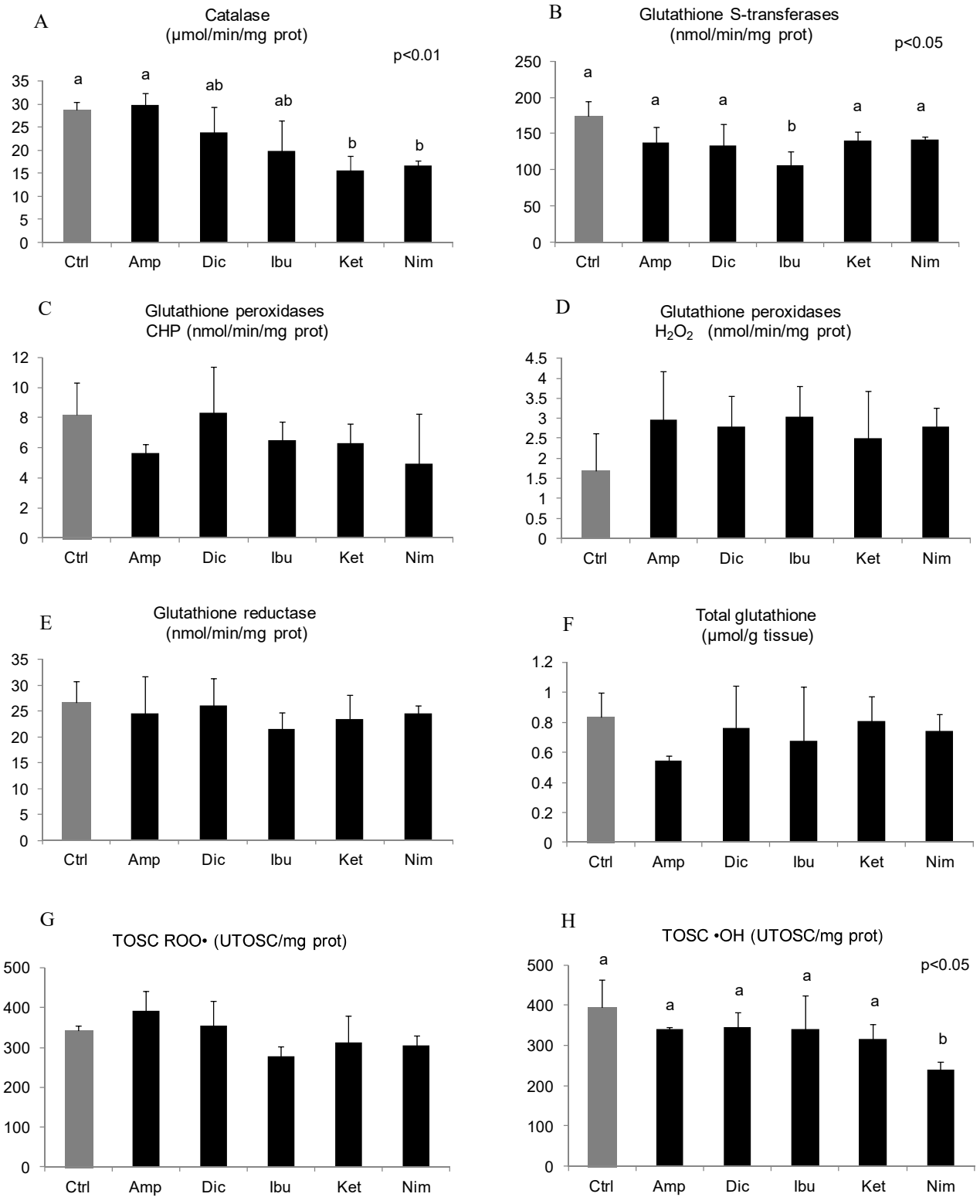

A
Catalase
(µmol/min/mg prot)
p<0.01
a
a
ab
ab
b
b
35
30
25
20
15
10
5
0
Ctrl
Amp
Dic
Ibu
Ket
Nim
B
Glutathione S-transferases
(nmol/min/mg prot)
p<0.05
250
200
150
100
50
0
C
Glutathione peroxidases
CHP (nmol/min/mg prot)
12
10
8
6
4
2
D
Glutathione peroxidases
H2O2 (nmol/min/mg prot)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
E
Glutathione reductase
(nmol/min/mg prot)
F
Total glutathione
(µmol/g tissue)
1.2
1
0.8
0.6
0.4
0.2
G
TOSC ROO• (UTOSC/mg prot)
500
400
300
200
100
H
TOSC •OH (UTOSC/mg prot)
p<0.05

Figure 2.

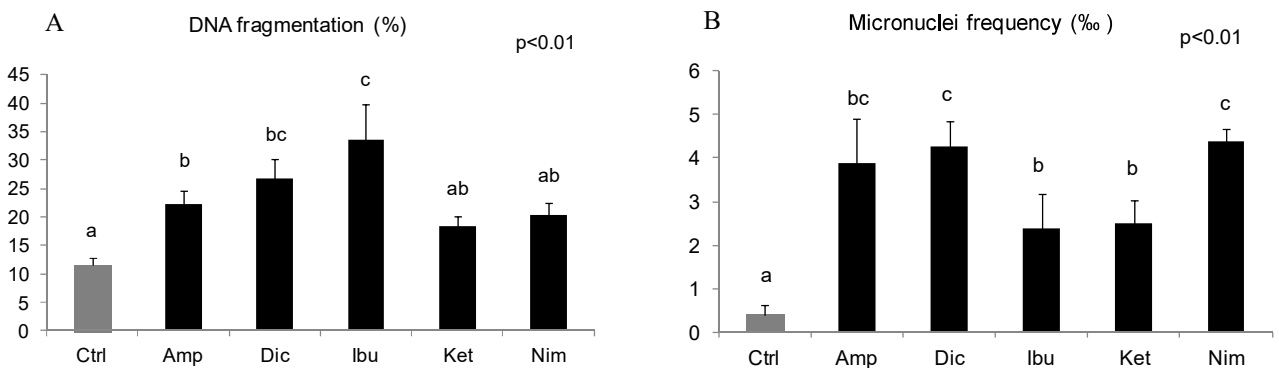

A
DNA fragmentation (%)
p<0.01
45
40
35
30
25
20
15
10
5
0
a
b
bc
c
ab
ab
Ctrl
Amp
Dic
Ibu
Ket
Nim
B
Micronuclei frequency (‰)
p<0.01
6
5
4
3
2
1
0
a
bc
c
b
b
c
Ctrl
Amp
Dic
Ibu
Ket
Nim

Figure 3.

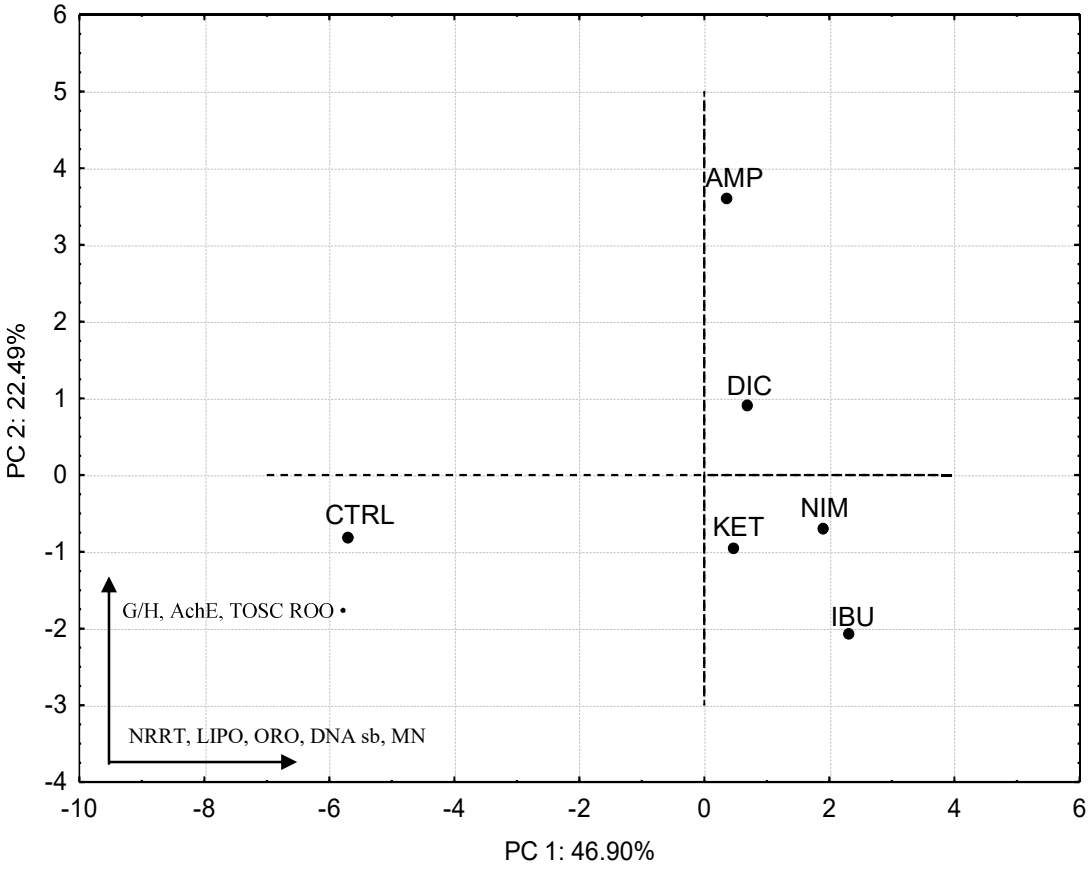

AMP
DIC
CTRL
KET
NIM
IBU
G/H, AchE, TOSC ROO •
NRRT, LIPO, ORO, DNA sb, MN
PC 2: 22.49%
PC 1: 46.90%
6
5
4
3
2
1
0
-1
-2
-3
-4
-10
-8
-6
-4
-2
0
2
4
6

Figure 4.

Supplementary Material

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