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

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note finali coverage

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

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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 become 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 (EMA) 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**

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

91 Haemocytes of the freshwater mussels *Dreissena polymorpha* revealed DNA fragmentation
92 and enhanced percentage of apoptotic cells when exposed to various doses of acetaminophen (30-
93 450 µg/L), diclofenac (60-250 µg/L) and ibuprofen (450-909 µg/L) (Parolini *et al.*, 2009). More
94 limited variations of the same parameters were reported after *in vivo* exposure of *D. polymorpha* to
95 lower concentrations of acetaminophen (0.75-1.51 µg/L) and ibuprofen (2-8 µg/L), along with
96 modulation of antioxidant enzymes (catalase, glutathione peroxidases and glutathione S-
97 transferases) and a decrement of lysosomal membrane stability (Parolini *et al.*, 2010, 2011).

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

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

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

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

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

140 2. Materials and Methods

141 2.1 Chemicals

142 Acetaminophen, AMP (CAS 103-90-2), Diclofenac, DIC (CAS 15307-86-5), Ibuprofen,
143 IBU (CAS 15687-27-1), Ketoprofen, KET (CAS 22071-15-4) and Nimesulide, NIM (CAS 51803-
144 78-2) were obtained from Sigma Aldrich (Milan, Italy); these chemicals were used for both the
145 exposure treatments and for analytical purposes.

146 2.2 Mussel exposure

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

154 The experimental design included five tanks with organisms exposed to 25 µg/L of AMP,
155 DIC, IBU, KET and NIM respectively and a solvent control tank (CTRL) where methanol was
156 added at the same concentration used in the NSAIDs treatments (0.003%). **The chosen exposure
157 concentration is higher than those typically found in marine field conditions (from a few units to
158 hundreds of ng/L; Gros et al., 2012; Lolić et al., 2015), but still environmentally realistic for
159 particularly challenged sites (up to tens of µg/L; Togola and Budzinski 2008).** Water was changed
160 daily, redosing concentrations of molecules and solvent; no mortality was observed during the
161 experiment. After 14 days, 30 specimens for each treatment were dissected for chemical analyses,
162 whole tissues were pooled in 5 samples (each containing tissues of 6 organisms) and stored at
163 -20°C. The remaining 30 specimens for each treatment were used for biological analyses preparing
164 10 replicates of haemolymph, digestive glands and gills, each constituted by tissues of 3 specimens.
165 Gills and digestive glands were frozen in liquid nitrogen and maintained at -80°C for the biomarker
166 analyses. Small pieces of digestive glands were also excised from the 10 specimens of each group
167 and frozen in hexane precooled to -70°C in liquid nitrogen, for histochemical analyses.
168 Haemolymph was withdrawn from the adductor muscle and divided in three aliquots: the first was
169 frozen in liquid nitrogen and maintained at -80 °C for the acetylcholinesterase analysis; another
170 aliquot was immediately processed for the analyses of neutral red retention time (NRRT),
171 granulocytes-hyalinocytes ratio, phagocytosis activity and DNA damage; the last aliquot of
172 haemolymph was fixed in Carnoy's solution (3:1 methanol, acetic acid) for the microscopic
173 evaluation of micronuclei frequency.

174 2.3 Field investigation

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

181 2.4 Chemical analyses

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

187 After centrifugation at 4500 ×g for 30 minutes, samples were purified by Solid Phase Extraction
188 (SPE) with reversed-phase tubes (Discovery DSC-18, 1g x 6 mL, Supelco, Bellefonte,
189 Pennsylvania, USA). SPE tubes were conditioned with 6 mL of methanol, followed by 18 mL of
190 ultra-pure water, and by 12 mL of buffer. Samples were loaded onto the SPE cartridges, and after
191 washing with 12 mL of ultra-pure water, analytes were eluted and recovered using 3 mL of
192 acetonitrile (HPLC, gradient grade, Carlo Erba). Obtained samples were filtered using PhenexTM-
193 RC membrane (Regenerated Cellulose/Polypropylene 0.45 μm, 15mm syringe filters, Phenomenex,
194 US) and then concentrated by SpeedVac at room temperature to a final volume of 1 mL.
195 Concentrated samples were centrifuged again at 12000 ×g for 20 minutes. Analytical detection of
196 extracted NSAIDs was performed by High Performance Liquid Chromatography, with fluorimetric
197 and diode array detectors DAD (Agilent Infinity 1260 series). Chromatographic separations of DIC,
198 IBU, KET and NIM were performed on a Kinetex column (C18, 5 μm, 150 mm length, 4.6 mmID,
199 Phenomenex, US), equipped with a security guard column (C18, 5 μm, 4 mm length, 2.0 mmID,
200 Phenomenex, US). For KET and DIC a mobile phase composed by ultra-pure water (26%),
201 acetonitrile (42%) and Buffer 1 (32%) was used under isocratic condition. DAD was used for
202 monitoring the spectra from 190 nm to 350 nm, and the signal for KET and DIC was obtained at
203 250 nm and 276 nm respectively. These wavelengths were selected among the spectra ranges
204 assuring at least the 85% of the maximum absorbance, verifying the absence of other spectral
205 interferences; additional qualifying signals (with about 50%-75% of the maximum absorbance)
206 were also recorded for each compound for the quality control and assurance. When necessary, the
207 full spectra was used to verify the purity of the obtained peaks, comparing them with those obtained
208 from pure standard solutions. Analyses of IBU and NIM were performed using ultra-pure water,
209 acetonitrile, Buffer 1 gradient (from 35%:30%:35% to 0%:65%:35% linearly during 23 minutes).
210 Analytical measurement of IBU was obtained by fluorimetric detector with excitation/emission
211 wavelengths at 230/294 nm, while NIM was detected using DAD from 190 nm to 410 nm and
212 monitoring at 298 nm. Also for this molecule, additional qualifying signals were obtained for
213 quality control and assurance and pure standard solutions were used to further verify the purity of
214 the obtained peaks. Separation of AMP was carried out by an Agilent Eclipse Plus column (C18,
215 3.5 μm, 100 mm length, 4.6 mmID) with a security guard column (C18, 5 μm, 4 mm length, 2.0
216 mmID, Phenomenex, US), and a mobile phase composed by Buffer 2 (87.5%) and methanol
217 (12.5%) under isocratic condition; a fast, post-run gradient was applied to remove any unresolved
218 compound retained by the analytical column (from 87.5%:12.5% to 10%:90%). Detection was
219 obtained using diode array in the range 190-350 nm, monitoring the signal at 248 nm and checking
220 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 ($\geq 98\%$).

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), granulocytes/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 Cochran 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**

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

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

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

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280 **3. Results**

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3 281 Mussels exposed to various NSAIDs showed a significant bioaccumulation of DIC, IBU and
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5 282 NIM, with tissue concentrations increasing from below detection limits up to 14.9 ± 7.89 ng/g, 1.63
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7 283 ± 1.00 ng/g, and 30.22 ± 13.50 ng/g d.w., respectively (Table 1). On the other hand, no variations
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9 284 were observed after 14 days for AMP and KET in mussels tissues, remaining below the limit of 1
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11 285 ng/g and 0.5 ng/g d.w., respectively (Table 1). Chemical analyses on wild mussels collected in the
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13 286 coastal area of Portonovo confirmed the lack of detectable accumulation of AMP and KET over the
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15 287 whole sampling period (Table 1). On the other hand, levels of DIC and IBU were below detection
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17 288 limit in July and September, while concentrations measured in August were comparable to those
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19 289 obtained after laboratory exposures for DIC and almost 6 folds higher for IBU (Table 1). NIM was
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21 290 always detected in wild organisms, with tissue concentrations rather comparable in different
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23 291 sampling months and lower compared to those obtained after laboratory exposures (Table 1).

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25 292 The analysis of biomarker responses in laboratory exposed mussels revealed that, among
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27 293 immunological parameters, lysosomal membrane stability was significantly reduced by all NSAIDs
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29 294 with more elevated effects in organisms exposed to DIC, IBU and NIM (Fig. 1A). Granulocytes-
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31 295 hyalinocytes ratio and phagocytosis activity did not exhibit any variation in exposed mussels (Fig.
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33 296 1B-C). A significant accumulation of lipofuscin in tertiary lysosomes occurred in all treatments,
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35 297 particularly in DIC and IBU exposed organisms (Fig. 1D), while only a limited, not significant
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37 298 decreasing trend was observed for neutral lipids (Fig. 1E). A marked inhibition of the acyl CoA
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39 299 oxidase activity was observed in organisms exposed to all the tested NSAIDs (Fig. 1F); the activity
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41 300 of acetylcholinesterase was significantly modulated only in haemolymph of organisms exposed to
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43 301 AMP, with no effect by other molecules and in gills (Fig. 1 G-H).

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45 302 Among antioxidant defences, catalase activity showed a significant inhibition after exposure
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47 303 to KET and NIM (Fig. 2A), while IBU caused the decrease of glutathione S- transferases (Fig. 2B).
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49 304 The exposure to NSAIDs did not cause any change in the activities of glutathione peroxidases (both
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51 305 Se-dependent and the sum of Se-dependent and Se-independent forms), glutathione reductase, and
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53 306 levels of total glutathione (Fig. 2C-F). The limited prooxidant effects induced by NSAIDs on
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55 307 exposed mussels were further supported by the analyses of the Total Oxyradical Scavenging
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57 308 Capacity, with a statistically significant decrease of TOSC only toward $\bullet\text{OH}$, for organisms exposed
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59 309 to NIM (Fig. 2 G-H).

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61 310 Genotoxic effects were revealed by the enhancement of DNA strand breaks particularly in
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63 311 IBU and DIC exposed organisms (Fig. 3A), and the marked increase of micronuclei frequency in
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65 312 mussels exposed to all NSAIDs (Fig. 3B).

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

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325 4. Discussion

326 Over the last fifteen years, pharmaceuticals have emerged as an important class of
327 environmental contaminants, with a documented occurrence in freshwater ecosystems, and potential
328 adverse effects on non target organisms (Parolini *et al.*, 2009; Santos *et al.*, 2010, Al Aukidy *et al.*,
329 2012). Relatively less attention has been paid to the ecotoxicological consequences of
330 pharmaceuticals released in the marine environment, despite recent investigations reported the
331 presence of about 113 pharmaceutical compounds in worldwide coastal waters at concentrations
332 ranging from 0.01 to 6800 ng/L (Gaw *et al.*, 2014).

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

340 Although various analytical methods have been reported for the determination of
341 pharmaceuticals in environmental abiotic matrices (Al Aukidy *et al.*, 2012; Gros *et al.*, 2012) more
342 limited protocols are available for their extraction and analysis in aquatic organisms (Huerta *et al.*
343 2013; Miller *et al.*, 2015). The successful development of specific HPLC procedures to quantify
344 NSAIDs concentration in mussels tissues allowed to characterize the capability of *M.*
345 *galloprovincialis* to accumulate the tested pharmaceuticals.

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

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

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

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

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

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

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

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

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

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

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

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

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

468
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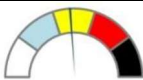




597 **TABLES**

598
599 **Table 1.** Bioaccumulation of acetaminophen (AMP), diclofenac (DIC), ibuprofen (IBU), ketoprofen
600 (KET) and nimesulide (NIM) in mussels, *M. galloprovincialis*, exposed in laboratory conditions
601 and in those sampled in field conditions. Data are given as ng/g d.w. (mean values ± standard
602 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

604 LOD: limit of detection

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609 **Table 2.** Quantitative Hazard Quotients (HQ) and assigned Class of hazard after weighted
610 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 \pm standard deviations, n=5). Different letters indicate significant differences between groups of means.

Figure 2. Antioxidant defenses and total oxyradical scavenging capacity (TOSC) toward peroxy (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 \pm 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 \pm 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 peroxy 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

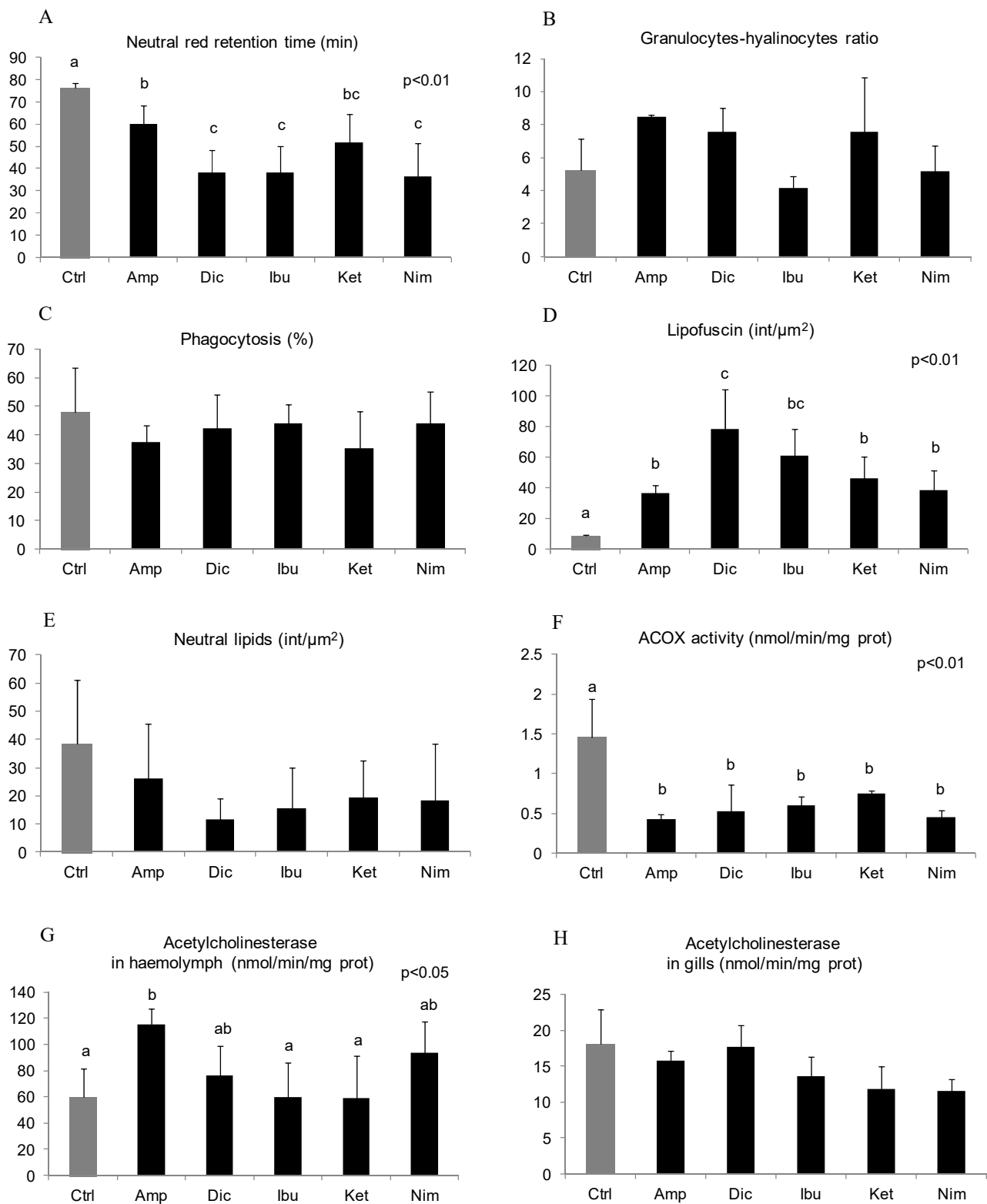


Figure 1.

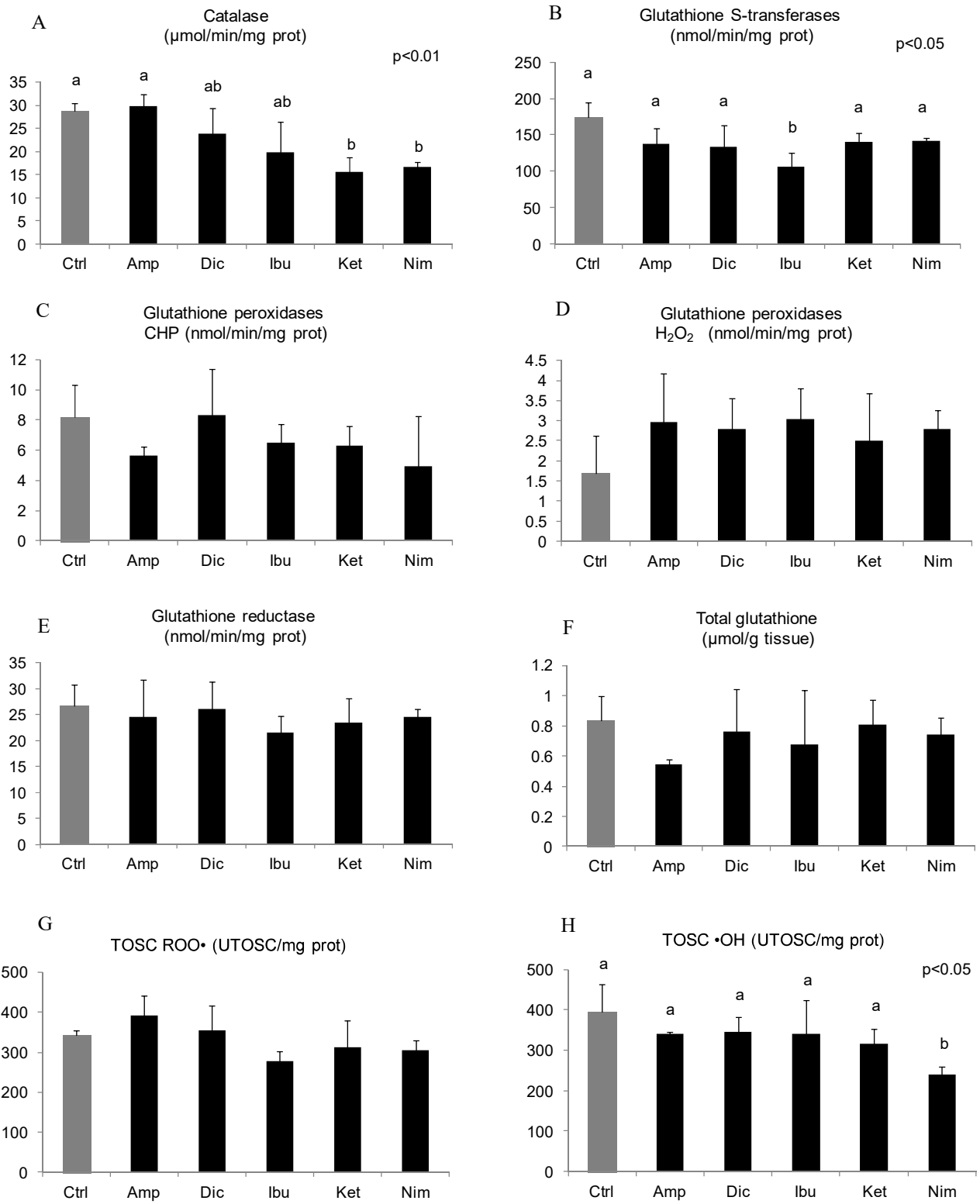


Figure 2.

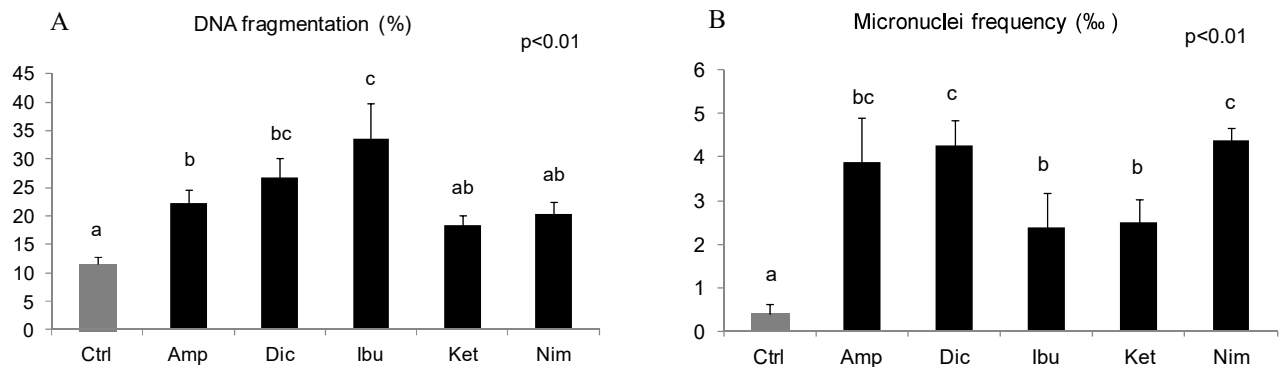


Figure 3.

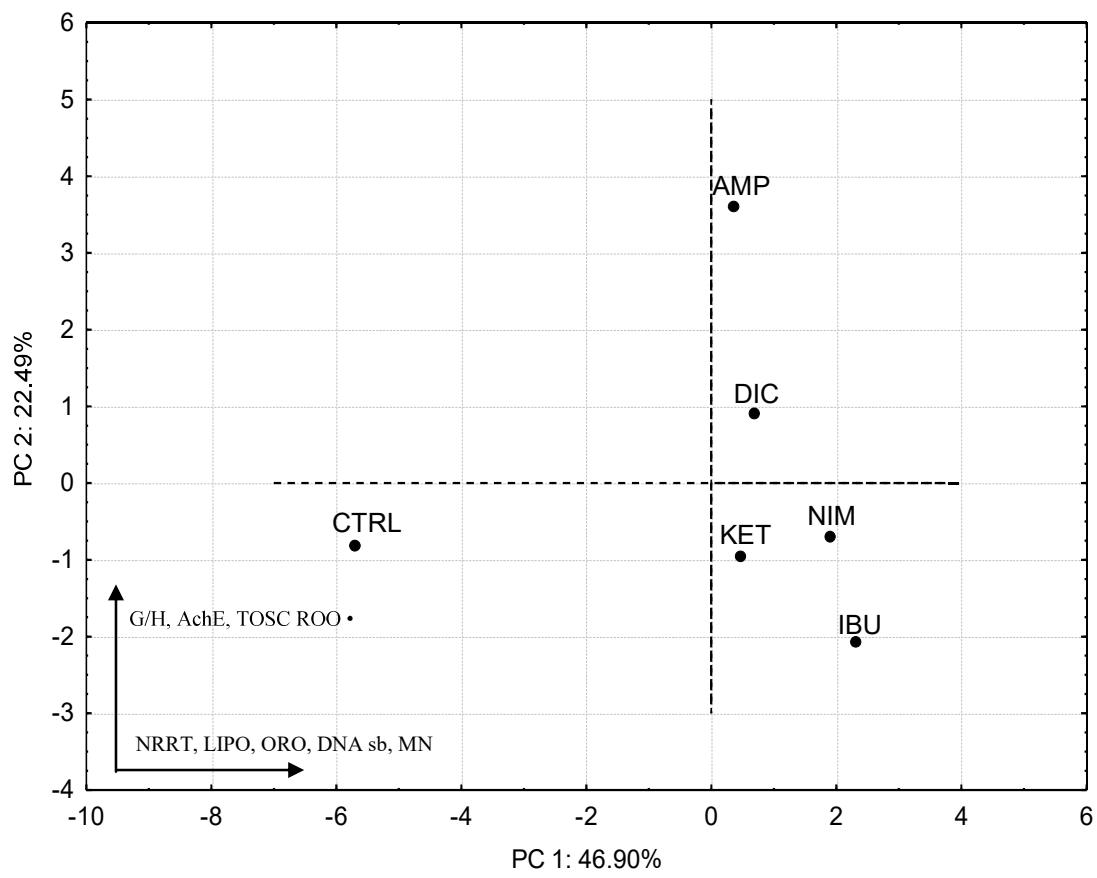


Figure 4.

Supplementary Material

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