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Long-term exposure of *Mytilus galloprovincialis* to diclofenac, Ibuprofen and Ketoprofen: Insights into bioavailability, biomarkers and transcriptomic changes.

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Abstract: Non-steroidal anti-inflammatory drugs (NSAIDs) represent a growing concern for marine ecosystems due to their ubiquitous occurrence and documented adverse effects on non-target organisms. Despite the remarkable efforts to elucidate bioaccumulation and ecotoxicological potential under short-term conditions, limited and fragmentary information is available for chronic exposures. In this study bioavailability, molecular and cellular effects of diclofenac (DIC), ibuprofen (IBU) and ketoprofen (KET) were investigated in mussels *Mytilus galloprovincialis* exposed to the realistic environmental concentration of 2.5µg/L for up to 60 days. Results indicated a significant accumulation of DIC and IBU but without a clear time-dependent trend; on the other hand, KET concentrations were always below the detection limit. Analyses of a large panel of molecular, biochemical and cellular biomarkers highlighted that all investigated NSAIDs caused alterations of immunological parameters, genotoxic effects, modulation of lipid metabolism and changes in cellular turn-over. This study provided the evidence of long-term ecotoxicological potential of NSAIDs, further unraveling the possible hazard for wild marine organisms.

HIGHLIGHTS

Long-term ecotoxicological effects of NSAIDs were investigated in mussels

Significant accumulation occurred for diclofenac, ibuprofen, not for ketoprofen

All NSAIDs determined transcriptional and cellular effects in mussels

Immune responses were the main targets of long-term exposure to NSAIDs

Transcriptional changes supported variations of cellular biomarkers

Biological alterations were maintained over 60 days of exposure

Università Politecnica delle Marche

Dipartimento Scienze della Vita e dell'Ambiente



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Ancona November 24th, 2017

Dr James Lazorchak
Toxicology and Risk Assessment,
Chemosphere

Mr Pallavi Das
Journal Manager
Chemosphere

Dear Dr James Lazorchak, dear Mr Pallavi Das,

thanks for your last message on the Crosscheck results, indicating a 23% of similarity (2,200 words) with a previous publication of my group (Mezzelani et al., "Transcriptional and cellular effects of Non-Steroidal Anti-Inflammatory Drugs, NSAIDs, in experimentally exposed mussels, *Mytilus galloprovincialis*" Aquatic Toxicology, 2016).

We have carefully revised the manuscript to verify such results and we noticed that such similarity is mostly related to Materials and Methods (approximately 1300 words) and References (approx. 900 words).

As Editor in Chief of an Elsevier Journal, I understand how difficult can be to interpret the results of the CrossCheck report in terms of originality or plagiarism. According to guidelines for interpreting CrossCheck similarity reports, an overall similarity index of 40% might be suspicious. However, a match may look artificially high due to standard phrasing, including references, or intentional use of specific phrases within a subject area (especially in Materials and Methods Section).

In our case, there is a valid reason for high similarity in this section, since the actual study compares experimental design and obtained results with those reported by Mezzelani et al., 2016. These 2 publications have in common the utilized biological model (the Mediterranean mussel, *Mytilus galloprovincialis*), the typology of many cellular analyses (those commonly performed on this species to evaluate the health status, or the effects of different stressors), and the biomarker elaboration: since this procedure is quite innovative and developed by us a few years ago, some explanations are considered useful for the reader.

In this respect, wording similarity derives from description of materials, methods and procedures which are quite standard and, in our opinion, adequately cited in the present submission.

We thus believe that wording similarity of our manuscript fallen well within the normal standard and, most importantly, it did not reflect any conceptual or scientific replication of an uncited previous study. Nonetheless, we made some minor changes on methods and presentation of the elaboration procedure (marked in red): should you still have any doubts, I

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would greatly appreciate a more detailed report/comments to make appropriate corrections (if any) or provide appropriate explanations (if any).

I hope that you can now consider the manuscript suitable for the reviewing process in Chemosphere.

Looking forward to hearing from you.

Yours sincerely,

Prof. Francesco Regoli

26 **Abstract**

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Non-steroidal anti-inflammatory drugs (NSAIDs) represent a growing concern for marine ecosystems due to their ubiquitous occurrence and documented adverse effects on non-target organisms. Despite the remarkable efforts to elucidate bioaccumulation and ecotoxicological potential under short-term conditions, limited and fragmentary information is available for chronic exposures. In this study bioavailability, molecular and cellular effects of diclofenac (DIC), ibuprofen (IBU) and ketoprofen (KET) were investigated in mussels *Mytilus galloprovincialis* exposed to the realistic environmental concentration of 2.5µg/L for up to 60 days. Results indicated a significant accumulation of DIC and IBU but without a clear time-dependent trend; on the other hand, KET concentrations were always below the detection limit. Analyses of a large panel of molecular, biochemical and cellular biomarkers highlighted that all investigated NSAIDs caused alterations of immunological parameters, genotoxic effects, modulation of lipid metabolism and changes in cellular turn-over. This study provided the evidence of long-term ecotoxicological potential of NSAIDs, further unraveling the possible hazard for wild marine organisms.

Keywords: Pharmaceuticals; Non Steroidal Anti-Inflammatory Drugs; marine mussels; long term exposure; bioaccumulation; biomarkers; DNA-microarray.

1. Introduction

Over the last few years, the presence of pharmaceuticals in aquatic ecosystems has been recognized as a crucial environmental issue. Occurrence in coastal areas was originally neglected, given the high dilution capacity of seawater, but recent studies demonstrated the presence of such compounds with concentrations ranging from a few ng/L to hundreds of $\mu\text{g/L}$ (Fabbri and Franzellitti, 2015; Lolić et al., 2015; Mezzelani et al., 2016). Adverse outcomes for marine organisms have been mostly investigated through standard tests or laboratory exposures in short-term conditions, typically from a few hours to 14 days (Aguirre-Martínez et al., 2015; Gonzales-Rey and Bebianno., 2011, 2012, 2014; Mezzelani et al., 2016a, b; Milan et al., 2013; Trombini et al., 2016). Although essential to establish acute toxicity, these data should be integrated with longer exposure studies to characterize the possible occurrence of chronic effects, with prognostic and diagnostic value on the impact of these ubiquitous molecules in marine biota (Cruz et al., 2016).

Among the most commonly used pharmaceuticals, non-steroidal anti-inflammatory drugs (NSAIDs) represent a proper example of a therapeutic class with worldwide environmental relevance: frequently measured in coastal areas (Lolić et al., 2015), they can be accumulated and interfere with cellular pathways in marine organisms (Bebianno and Gonzalez-Rey, 2015; Mezzelani et al., 2016a,b). In previous studies, the Mediterranean mussel *Mytilus galloprovincialis* was exposed for 14 days at environmentally realistic concentrations of different NSAIDs (0.5-25 $\mu\text{g/L}$): these organisms demonstrated the capability to accumulate Diclofenac (DIC), Ibuprofen (IBU) and Nimesulide (NIM) with a dose-dependent response, while Acetaminophen (AMP) and Ketoprofen (KET) were never detectable in mussels tissues (Mezzelani et al., 2016a,b). All NSAIDs, however, determined the onset of biochemical and cellular alterations with effects of greater magnitude at the higher doses of exposure: immunological functions, lipid metabolisms and cellular turnover appeared as the primarily modulated responses. These effects were confirmed at molecular level for mussels exposed to 0.5 $\mu\text{g/L}$ of KET and NIM, with significant changes of

67 transcriptional profile and expression of numbers of genes involved in eicosanoids metabolism,
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transcriptional profile and expression of numbers of genes involved in eicosanoids metabolism, immune system, apoptosis, cell-cycle regulation, reproduction and energetic metabolism (Mezzelani et al., 2016b). Biochemical and cellular effects of DIC and IBU in *M. galloprovincialis* were demonstrated also at a lower exposure concentration (0.25 µg/L), with a transitory induction of antioxidant enzymes and increase of lipid peroxidation after 14-days (Gonzales-Rey and Bebianno., 2011, 2012, 2014).

67 In addition to laboratory experiments, recent studies documented the presence of anti-inflammatory
68 drugs in tissues of wild marine organisms sampled along Italian and Spanish coasts (Mezzelani et
69 al., 2016a; Moreno-González et al., 2016). These evidences corroborate the hypothesis that aquatic
70 organisms are challenged by continuous release of pharmaceuticals in natural ecosystems raising
71 the need to elucidate whether chronic exposure conditions further exacerbate molecular,
72 biochemical and cellular outcomes in non target species (Peters and Granek, 2016).

73 In this respect, the present study investigated the long-term ecotoxicological potential of
74 DIC, IBU and KET in *M. galloprovincialis* exposed for up to 60 days to 2.5 µg/L, an
75 environmentally realistic level, similar to concentrations possibly occurring in coastal areas
76 (Castiglioni et al., 2005; Loos et al., 2013; Togola and Budzinki, 2008; Zuccato et al., 2006). The
77 work was based on a multidisciplinary approach integrating measurement of drug bioaccumulation
78 with a large number of biomarkers reflecting the perturbation of different cellular districts and
79 molecular pathways. Such responses included alteration of immunological parameters, lipid and
80 oxidative metabolism, onset of genotoxic effects. Considering the urgency of prioritize active
81 pharmaceuticals discharged in aquatic environment (Boxall et al., 2012), the biological relevance of
82 observed variations has been elaborated through a quantitative model and weighted criteria
83 (Sediqualsoft) which summarize a cellular hazard index based on the toxicological importance of
84 analyzed biomarkers, number and magnitude of measured effects (Benedetti et al., 2012; Piva et al.,
85 2011).

92 Gene transcriptional profile was further analyzed in KET exposed mussels since this molecule was
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23 already shown to cause significant effects after 14 days of treatment, independently on the lack of
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54 bioaccumulation (Mezzelani et al., 2016b). In this respect, an additional objective of this study was
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75 to investigate whether changes in gene expression can be considered as suitable biomarkers of
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96 NSAIDs exposure also in long-term conditions, or if they rather represent transitory responses
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127 before more integrated effects occur at cellular level.
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1598 The overall results of this study were expected to provide new insights on the potential risk of
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1799 constant exposure to NSAIDs in marine organisms contributing to elucidate links between cellular
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100 effects and ecological consequences of pharmaceuticals in coastal areas.
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202 **2. Materials and methods**

203 2.1 Experimental design

204 Stock solutions of Diclofenac, Ibuprofen and Ketoprofen (Sigma Aldrich) were stored at room
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105 temperature (10 mg/L in methanol) and diluted in seawater during exposures of mussels.
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3406 Control organisms, *M. galloprovincialis* (5 ± 1 cm shell length), obtained from an unpolluted area
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307 of Central Adriatic Sea (Portonovo, Ancona), were maintained for 10 days at constant laboratory
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308 conditions (aerated seawater, $18 \pm 1^\circ\text{C}$, salinity 37, pH 8.0 ± 0.5 and oxygen saturation $>94\%$).
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4109 Mussels were randomly sub-divided in twelve 20 L aquarium (60 mussels per tank, for a total of
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44720 specimens). The experimental design included nine tanks with organisms exposed to $2.5 \mu\text{g/L}$
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4611 of DIC, IBU and KET respectively (3 tanks for each treatment), and three solvent control tanks
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49 (CTRL) where methanol was added at the same concentration used in NSAIDs treatments
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5113 (0.003%). The water in each tank was daily changed and concentrations of various drugs were re-
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5414 established afterwards. Before water change, mussels were supplied with food (Zooplanktös-sTM,
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5615 50-300 μm), and no mortality was observed during the experiment. At days 14, 30 and 60, whole
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5916 tissues were removed from 30 specimens for treatment (10 from each tank), pooled in 5 samples
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617 (each constituted by tissues of 6 organisms) and stored at -20°C until chemical analyses. Moreover,
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118 haemolymph, digestive glands and gills were immediately dissected from the remaining 30
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119 specimens: 10 pools (each with tissues of 3 mussels) were frozen in liquid nitrogen and stored at -
3
120 80°C until biochemical and histochemical analyses. Lysosomal membrane destabilization,
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121 phagocytosis and loss of DNA integrity (Comet assay) were immediately processed in haemocytes
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122 using an aliquot of fresh haemolymph; another aliquot of chemically fixed haemolymph (Carnoy's
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123 solution, 3:1 methanol, acetic acid) was microscopically analyzed for granulocytes-hyalinocytes
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124 ratio and micronuclei frequency.
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125 Samples for DNA microarray analysis were also prepared from CTRL and KET exposed mussels
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126 (four pools, each with digestive glands of three organisms).
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2.2 Chemical analyses

129 Protocols for measuring tissues concentrations of NSAIDs in exposed mussel by high performance
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29 liquid chromatography, HPLC, were recently validated (Mezzelani et al., 2016a). For each group,
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31 the five prepared pools were homogenized (1:1.6 w:v) in acetic acid 0.1%, pH=3.26 (buffer 1) at
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33 room temperature for 20 minutes, and then centrifuged at 4,500 xg for 30 minutes. Samples were
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35 purified by Solid Phase Extraction (SPE), using tubes pre-conditioned with methanol, ultra-pure
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37 water and buffer, and recovering analytes with acetonitrile. After filtration and concentration by
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39 SpeedVac, obtained samples were centrifuged again at 12,000 xg for 20 minutes.

43 Separations of KET and DIC were performed under isocratic condition with a mobile phase
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45 composed by ultra-pure water (26%), acetonitrile (42%) and Buffer 1 (32%), elaborating
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47 chromatographic signals by diode array detector (DAD) at 250 nm and 276 nm, for KET and DIC
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49 respectively. IBU was separated on a gradient using ultra-pure water, acetonitrile and buffer 1 (from
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51 35%:30%:35% to 0%:65%:35% for 23 minutes), and detected by fluorimetric detector (FD) with
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53 excitation/emission wavelengths at 230/294 nm. Pure standard solutions of various NSAIDs were
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55 used to quantify concentrations measured in mussels' tissues. Considering the absence of Standard
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57 Reference Materials (SRMs), a series of blank samples were spiked with various doses of pure

144 NSAIDs standards to assess the reproducibility, quality assurance and quality control of analytical
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145 procedures (Mezzelani et al., 2016a). The recovery yield was always $\geq 98\%$ ($CV < 5\%$, $n=10$), with
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146 an instrumental limit of detection (LOD) in mussel tissues of 1 ng/g dry weight (d.w.) for DIC and
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147 0.5 ng/g (d.w.) for IBU and KET.
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149 2.3 Biological responses

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150 Validated protocols were used to analyze the following biomarkers in tissues of exposed mussels:

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151 immunological responses as lysosomal membrane stability (neutral red retention time, NRRT),

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152 granulocytes/hyalinocytes ratio (G/H) and phagocytosis activity in haemocytes; peroxisomal

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153 proliferation (Acyl-CoA oxidase, ACOX) and neurotoxic effects (acetylcholinesterase, AChE) in

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154 haemolymph and gills; antioxidants defences and susceptibility to oxidative stress in digestive

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155 gland (catalase CAT, glutathione S-transferases GST, Se-dependent glutathione peroxidases GPx

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156 H₂O₂, total GPx CHP, glutathione reductase GR, total glutathione GSH, total antioxidant capacity

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157 TOSC toward peroxy radicals ROO• and hydroxyl radicals HO•); accumulation of lipid

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158 peroxidation products (lipofuscin, LIPO) and neutral lipids (NL) in digestive gland; loss of DNA

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159 integrity (DNA strand breaks and micronuclei, MN) in haemocytes. Detailed protocols are given in

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160 Supplementary Material 1 (SM1).

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162 2.4 Labelling, microarray hybridization and data acquisition

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163 Transcriptional profile was analyzed using an 8x60K Agilent oligo-DNA microarray in CTRL and

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164 KET groups, using 4 pools of digestive glands, each containing tissues of 3 individuals. This

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165 platform, developed within the REPROSEED European project (REsearch project to improve

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166 PROduction of SEED of established and emerging bivalve species in European hatcheries) contains

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167 59,997 probes, representing a total of 50,680 contigs for *M. galloprovincialis*. Details on the

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168 microarray and probe sequences are given in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>,

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169 accession number GPL18667) while other detail on sequencing, assembly, annotation and

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170 microarray design are summarized in Supplementary Material 1 (SM1). Microarrays were
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171 synthesized through the *in situ* non-contact ink-jet technology including default positive and
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172 negative controls, and to the Agilent One-Color Microarray-Based Gene Expression Analysis
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173 protocol was used for sample labelling and hybridization with the Low Input Quick Amp Labeling
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174 kit. Labelling, hybridization and data acquisition procedures are detailed in Supplementary Material
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125 2 (SM2).

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176 Normalization procedures included quantile normalization, which always outperformed cyclic
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177 loess. Data on raw gene expression and normalized data can be accessed in the GEO database
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178 (GSE103295 and GSE103295, respectively).

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2.5 Statistical analyses

181 Analysis of variance was applied to test differences between treatments and exposure days (level of
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significance at $p < 0.001$). Cochran C was applied for testing homogeneity of variance, with
appropriate mathematical transformation if necessary; the Student Newman-Keuls test, (SNK) was
used for post-hoc comparison between means of values ($n=5$). Statistical analyses were performed
using R-cran software (<http://www.R-project.org>).

The overall biological significance of biomarker responses was summarized in a hazard index with
a quantitative, software-assisted model (Sediqualsoft), which uses weighted criteria to elaborate
large data-sets of heterogeneous data (Benedetti et al., 2012, 2014; Piva et al., 2011). Briefly, all
biomarkers have a specific “weight”, depending on the toxicological relevance of the endpoint, and
a “threshold” which defines the minimum percentage of variation considered of biological
significance. Each biomarker response is compared to the respective threshold, corrected for both
the statistical significance of the variation and the toxicological weight of the response: detailed
flow-charts, rationale for weights, thresholds and expert judgements have been fully given
elsewhere (Benedetti et al., 2012, 2014; Piva et al., 2011). The Hazard Quotient for biomarker
variations (HQ_{BM}) is calculated without considering the Effects lower or equal to respective

196 thresholds, averaging those with an effect up to two-fold compared to the threshold, and adding the
 197 summation (Σ) for the biomarker responses more than 2 fold greater than the respective threshold
 198 (Benedetti et al., 2012, 2014; Piva et al., 2011):

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

201 The calculated HQ_{BM} is finally attributed to one of five classes of cumulative hazard for biomarkers
 202 (from Absent to Severe), maintaining scientifically sound information, while also being easy to read
 203 for non-expert stakeholders (Benedetti et al., 2012, 2014; Piva et al., 2011).

204 Transcriptional results were statistical analyzed selecting 45,502 out of 59,971 probes with signal
 205 higher than the background in at least 4 out of 24 analysed samples. Principal Component Analysis
 206 (PCA) and T-test statistics (Bonferroni correction for multiple comparisons; p-value<0.05; 200
 207 permutations) elaborated results obtained from CTRL and KET exposed mussels at each time, 14,
 208 30 and 60 days (TIGR Multi Experiment Viewer, TMeV 4.5.1 statistical software, by Saeed et al.,
 209 2003).

210 Only probes with fold change (FC) > 1.5 and signal higher than the background in at least one
 211 group have been considered as differentially expressed genes (DEGs). Functional connections of
 212 observed DEGs were investigated using Database for Annotation, Visualization, and Integrated
 213 Discovery (DAVID) software (Huang et al., 2009), matching transcripts of *M. galloprovincialis* to
 214 *Danio rerio* Gene IDs using dedicated Blast searches performed with blastx (E-value < 10⁻⁵). Since
 215 functional annotation data are available for a limited number of species, the choice of *D. rerio* was
 216 previously demonstrated to allow the assignment of a putative homologue to a larger number of
 217 transcripts in bivalves, such as *M. galloprovincialis* (Avio et al., 2015), and *Ruditapes*

219 *philippinarum* (Milan et al., 2011, 2013). Genes differentially expressed in each T-test pairwise
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220 comparison, were functionally annotated setting DAVID for gene count=2 and ease=0.1.
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222 **3. Results**

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223 Levels of NSAIDs in control and exposed mussels are reported in Table 1. While values were
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224 always below the instrumental limit of detection (LOD) in controls, an increase of tissue
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225 concentrations was observed in DIC and, particularly, in IBU-exposed organisms. In both the
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226 treatments, values measured after 14 days did not statistically change over the following exposure
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1227 period, despite mean values of IBU in mussels tissues almost doubled at day 60 compared to
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228 previous times. Tissue levels of KET were always below LOD.

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1229 Results of analyzed biomarkers are reported in Table 2, while those of two-way analysis of
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230 variance (ANOVA) are given in Table 1 of Supplementary Material 1 (SM1). To facilitate the
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231 comparison between the effects caused by different NSAIDs at various days of exposure,
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232 biomarkers with statistically significant changes are also shown as percentage variations compared
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233 to relative control organisms (Figure 1).

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234 Immunological responses were evident as reduction of lysosomal membrane stability in mussels
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235 exposed to IBU and KET after 14, 30 and 60 days; the same trend, although not statistically
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236 significant, was observed for DIC treatment (Fig. 1A). The granulocytes/hyalinocytes ratio was
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237 decreased by all tested NSAIDs (Fig. 1B): time dependent effects were measured for DIC and IBU,
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238 while the sharp reduction caused by KET after 14 days remained almost constant during the
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239 following exposure period. Phagocytosis capacity exhibited an inhibition trend for all treatments
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240 that, however, resulted statistically significant only for IBU and KET after 30 days of exposure
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241 (Fig. 1C).

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242 Lipofuscin content in tertiary lysosomes appeared markedly enhanced only in organisms exposed to
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243 KET after 60 days. (Fig. 1D), while neutral lipids significantly increased after 30 days in organisms
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244 treated with IBU and KET (Fig. 1E). The activity of acyl CoA oxidase tended to be decreased by
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245 various NSAIDs with a significant effect in KET-exposed organisms after 30 days (Fig. 1F).

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246 The measurement of genotoxic effects revealed a generalized increase of DNA strand breaks with
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247 significantly higher values in mussels exposed to DIC for 14 and 60 days and to KET for 14 days

248 (Fig. 1G). Levels of micronuclei exhibited a marked, but transitory enhancement for all the
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249 treatments, specifically after 30 days for DIC, after 14 and 30 days for IBU and KET (Fig. 1H).
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250 No significant variations were measured for acetylcholinesterase in both haemolymph and gills at
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251 various treatments and exposure period, and antioxidant defenses (catalase, glutathione reductase,
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252 glutathione peroxidases, glutathione S-transferases and levels of glutathione) also showed only
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253 limited and not statistically significant variations between the different experimental conditions
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254 (Table 2). The lack of prooxidant effects induced by anti-inflammatory pharmaceuticals in mussels
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255 was further supported by the results on Total Oxyradical Scavenging Capacity with almost constant
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256 values toward both peroxy and hydroxyl radicals (Table 2).
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257 Considering the biological relevance (weight) and magnitude of observed effects, the elaboration of
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258 biomarker results with weighted criteria summarized as respectively “Slight” and “Moderate” the
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259 hazard for organisms exposed to DIC and IBU over the entire exposure period. In KET exposed
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260 organisms, the level “Slight” obtained after 14 days increased to “Moderate” after 30 and 60 days of
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261 treatment (Table 3).
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262 Transcriptional changes in KET exposed mussels, highlighted a total of 674, 469 and 447
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263 differentially expressed genes (DEGs) at 14, 30 and 60 days, respectively (Figure 2); the full list of
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264 DEGs is reported in Supplementary Material 2 (SM2). The Venn diagram revealed that a unique
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265 probe, coding for putative cAMP responsive element binding protein-like 2 (CREBL2), was
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266 significantly down-regulated at all times of exposure (Fig.2). Other DEGs included transcripts
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46
267 involved in the arachidonic acid (AA) metabolism (*phospholipase A2 PLA2*, *arachidonate 5-*
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48
268 *lipoygenase ALOX5*), immune responses (*toll-like receptors TLR2, TLR4, TLR4B, TLR 7, TLR21,*
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269 *Rhamnose-binding lectin RBL, hepatic lectin HLEC*), lipid metabolism (*hydroxyacyl-Coenzyme A*
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270 *dehydrogenase ACOT13, Acyl-Coenzyme A thioesterase HADH, acetyl-Coenzyme A acyltransferase*
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271 *1 ACAA1*), cell cycle (*Cyclin-I CCN1, Cell division cycle protein 123 CDC 123, cell division cycle*
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272 *14 homolog A CDC14*), apoptosis (*baculoviral IAP repeat-containing 2 BIRC2, baculoviral IAP*
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273 *repeat-containing 7 BIRC7, X-linked inhibitor of apoptosis XIAP, caspase 3 CASP3, DNA-damage*

274 *regulated autophagy modulator 2a DRAM2, DNA fragmentation factor subunit beta DFFB*) and
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275 DNA damage (*DNA-damage inducible protein DDII, nucleotide excision repair homolog MMS19,*
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276 *UV excision repair protein RAD23, growth arrest and DNA-damage-inducible alpha GADD45A*).

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277 The enrichment analysis with DAVID software (Dennis et al. 2003; Huang et al. 2009) provided a
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278 more systematic functional interpretation of observed DEGs. This analysis has been performed
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1279 separately for down- and up-regulated genes in response to KET exposure. Lists of significantly
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280 enriched Biological Process (BP), Cellular Component (CC), Molecular Function (MF) GO terms
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281 and KEGG pathways are fully reported in Supplementary Material 3 (SM3), and summarized in
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282 Table 4. The most represented enriched terms included “endocytosis” and “oxidation reduction” (at
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21
283 14 and 30 days), “apoptosis” (at 14 and 60 days), “RNA processing” (at 14 days), “macromolecule
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284 catabolic process”, “NOD-like receptor signaling pathway” (at 30 and 60 days), “fatty acid
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285 metabolic and biosynthetic process” (at 30 days), and “Toll-like receptor signaling pathway” (at 60
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286 days).

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288 **4. Discussion**

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The present investigation aimed to study the long-term ecotoxicological potential of NSAIDs in *M. galloprovincialis*, providing novel insights on possible adverse effects and hazard in coastal areas. Pharmaceuticals should be considered as high priority environmental pollutants (Aguirre-Martínez et al., 2016; Bebianno and Gonzalez-Rey, 2015; Daughton, 2016; Fabbri, 2015; He et al., 2017) and a particular concern has been raised for NSAIDs, one of the most consumed therapeutical class worldwide, with ubiquitous occurrence in aquatic ecosystems (Brune and Patrignani, 2015; He et al., 2017; Kim et al., 2015; Paíga et al., 2013). Such compounds were elected as ideal candidates for eco-pharmacoavilance investigations aimed to characterize the distribution, adverse effects and fate of pharmaceuticals in the environment to implement knowledge-based reduction and prevention measures (He et al., 2017; Holm et al., 2013; Nieto et al., 2017). In this respect, physic, chemical and biological properties of NSAIDs should all be considered when assessing their

300 environmental impact (McEneff et al., 2014). In fact, although susceptible to degradation and with a
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301 half-life usually shorter than other pollutants, the continuous introduction into the aquatic
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302 environment actually confers to NSAIDs pseudo-persistence characteristics (Ankley et al., 2007;
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303 Hernando et al., 2006). In our study, mussels exposed to the environmentally realistic concentration
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304 of 2.5µg/L for 60 days, showed measurable levels of DIC and IBU, after 14 days without a clear
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305 time-dependent increase at longer exposure periods: a similar result may suggest the activation of
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306 detoxification pathways or excretion processes leading to a steady state of NSAIDs tissue
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307 concentrations. These results confirmed the possibility of marine mussels to accumulate
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308 pharmaceuticals over a wide range of environmental levels: the comparison of tissue levels
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309 measured in this study with those observed in organisms treated with 0.5 or 25µg/L of the same
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310 compounds, highlight a possible dose dependent behavior for DIC, but not for IBU (Mezzelani et
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311 al., 2016a,b), underling that molecules with similar mechanisms of action do not have the same
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312 pharmacokinetic pathways. This aspect is further corroborated by the lack of accumulation of KET,
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313 always below the LOD, independently from the exposure times and concentrations used in the
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314 present or in the previous investigations (Mezzelani et al., 2016a,b). Considering the onset of
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315 biological effects in organisms exposed to this drug, it has already been suggested that the lack of
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316 KET accumulation in mussels tissues probably reflect the transformation of this molecule either in
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317 the environment or within cells once accumulated. Worthy to note, levels of anti-inflammatory
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318 drugs measured in laboratory experiments are comparable with those measured in wild mussels
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319 from the Italian coasts (Mezzelani et al., 2016a) with similar concentrations for DIC and IBU, while
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320 never detectable for KET.

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321 Among the wide panel of analyzed biological responses, lysosomal membrane stability was
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322 confirmed as a sensitive biomarker for detecting adverse effects of anti-inflammatory compounds,
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323 with an early responsiveness at 14 days, then constantly maintained over 60 days of exposure. The
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324 comparison with previous experiments performed in a wider range of environmentally realistic
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325 concentrations (0.5-2.5-25µg/L) (Mezzelani et al., 2016a,b) reveal a dose dependent modulation of

326 this parameter for DIC and KET, while IBU appeared to strongly reduce lysosomal stability at all
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327 the doses of exposure. This study further supported the modulation of immune system with the
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328 significant decrease of granulocytes/hyalinocytes ratio and the parallel inhibition of phagocytosis
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329 capacity: the latter effect is presumably related to the lower phagocytic capacity of hyalinocytes
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330 compared to granulocytes which normally represent the dominant cell type in haemolymph of *M.*
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331 *galloprovincialis* (Gorbi et al., 2012, 2013), responsible for cell-mediated immunity through
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332 phagocytosis and cytotoxic reactions (Ciacci et al., 2012). Despite precise mechanisms behind the
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333 modulation of these pathways remain unclear, the present study unravels the suitability of immune
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334 system biomarkers as targets of long-term exposure to anti-inflammatory compounds. Antioxidant
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335 biomarkers generally showed quite constant values of catalase, glutathione dependent enzymes and
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336 level of total glutathione. The lack of oxidative challenge determined by NSAIDs was further
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337 confirmed by the total oxyradical scavenging capacity toward peroxy and hydroxyl radicals which
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338 were never affected by any experimental condition. These findings, in agreement with previous
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339 short-term investigations on marine bivalves, confirm that prooxidant mechanisms do not represent
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340 the primary mode of action of these pharmaceuticals neither in short, nor in long-term exposure
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341 conditions (Bebiano and Gonzalez-Rey, 2015; Bebianno et al., 2017; Mezzelani et al., 2016 a, b).
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342 The limited oxidative challenge of NSAIDs was reflected by results obtained on lipofuscin, an
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343 intralysosomal non degradable product, primarily composed of cross-linked proteins and lipids
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344 accumulated under oxidative stress conditions (Regoli, 1992). An appreciable increment was
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345 measured in KET exposed mussels only at the end of the treatment while a more generalized
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346 accumulation of lipofuscin had been previously documented after 14 days of exposure to higher
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347 levels of NSAIDs (Mezzelani et al., 2016a,b). Variable and fluctuating results on lipofuscin content
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348 are quite common in marine organisms exposed to active pharmaceutical ingredients (Gonzalez-
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349 Rey et al., 2014; Nunes et al., 2017): unless challenged by prooxidant chemicals, lipofuscin can be
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350 modulated by various biological factors including reproductive cycle, feeding activities and
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351 autophagic processes to cite a few. In this study, a transient increase of neutral lipids was observed
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352 in mussels exposed to NSAIDs, contrasting with recent investigations showing a high degradation
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353 of such energy reserves, possibly related to a direct modulation of lipid metabolism mediated by the
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354 drugs (Mezzelani et al., 2016a,b). However, it should be noted that control organisms had
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355 particularly low basal levels of neutral lipids which reflected the pronounced seasonal pattern of
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356 these reserves with minimum levels during autumn and winter season when glycogen is mostly
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357 utilized (Regoli et al., 1992). In this respect, the present study revealed slight changes also in the
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358 activity of the Acyl CoA oxidase (ACOX) which catalyzes the first reaction of β -mitochondrial
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359 oxidation of fatty acids revealing a potential change in metabolisms of energy resources, only in
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360 KET exposed mussels. Long-term exposure to anti-inflammatory drugs determined limited
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361 fluctuations on the AchE activity, which has not been highlighted as a main target of NSAIDs also
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362 in previous investigations on mussels, showing a moderate induction in gills after 3 and 7 days of
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363 exposure to 250 ng/L of DIC (Gonzalez-Rey and Bebianno, 2014, Mezzelani et al., 2016a,b).
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364 Genotoxic damages expressed as DNA fragmentation and frequency of micronuclei tended to
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365 increase in mussels response to all investigated compounds with time dependent enhancement of
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366 DNA strand breaks for DIC and IBU exposed mussels. The frequency of micronuclei over 60 days
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367 of exposure showed a typical biphasic trend for all investigated drugs, corroborating the hypothesis
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368 that NSAIDs would not increase MN acting as direct genotoxic compounds on DNA integrity, but
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369 rather influencing the haemocytes cellular turn-over (Chang et al., 2009; Mezzelani et al., 2016a,b).
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370 The elaboration of all biomarkers data within the Weight Of Evidence model suggest that biological
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371 responses quickly reach their steady state conditions in mussels exposed to DIC and IBU where the
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372 summarized hazard indices were “Slight” and “Moderate” after 14 days, then remaining constant
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373 for the entire duration of exposure; only in KET exposed organisms the hazard level elaborated as
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374 “Slight” after 14 days increased to “Moderate” at 30 and 60 days of treatment. These results
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375 confirm that, despite concentrations of KET in mussels tissues were always below the LOD,
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376 biological effects are evident and tend to increase in chronic conditions, reinforcing the hypothesis
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377 on bioavailability and potential (bio)-transformation of this drug (Mezzelani et al., 2016a,b).
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378 To further corroborate this possibility, transcriptomic profile was analyzed in KET exposed
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379 mussels. Combining cellular biomarkers responses with transcriptomic analysis is of particular
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380 relevance for emerging contaminants, to obtain new insights on their mechanisms of action (MOA).
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381 Since aquatic wildlife may possess conserved targets related to the drug metabolism,
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382 pharmaceuticals are potentially able to alter essential homeostatic mechanisms even at
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383 concentrations lower than those for human dosage (Fabbri and Franzellitti, 2015; Maria et al.,
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384 2016).

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385 Overall, the number of DEGs was more elevated after 14 days (674) compared to 30 and 60 days
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386 (469 and 477 DEGs, respectively), which may suggest that cellular response is initially
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387 characterized by a generalized early modulation of several pathways while, at more prolonged
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388 exposure, a higher specificity of DEGs would prevail. In mammals, NSAIDs inhibit the reaction
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389 catalyzed by the cyclo-oxygenase enzymes COX-1 and COX-2, preventing the formation of
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390 prostaglandins and thromboxane from arachidonic acid (Gierse et al., 1995; Parolini et al., 2011). In
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391 this work, transcriptional profiles provided evidence on the effects of KET toward arachidonic acid
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392 (AA) metabolism in mussels. PLA2 is a key enzyme in catalyzing the hydrolysis of cellular
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393 phospholipids at the sn-2 position to liberate AA during eicosanoids metabolism; its up-regulation
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394 after 60 days of KET exposure may indicate a potential recruitment of AA to increase the substrate
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395 availability as a result of COX inhibition. This finding confirmed the results obtained in mussels
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396 exposed to 0.5µg/L of KET (Mezzelani et al., 2016 b) and in Manila clam exposed to IBU (Milan et
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397 al. 2011), further highlighting direct ecotoxicological effects of NSAIDs on AA metabolism with
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398 potentially deleterious consequences in reproduction, gametogenesis and larval settlement (Knight
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399 et al., 2000; Stanley, 2000).

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400 Molecular analyses strongly supported variations observed for cellular biomarkers, in particular
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401 those on immune responses with changes in a large number of genes regulating toll-like receptors,
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402 and lectins (*TLR2*, *TLR4*, *TLR4B*, *TLR7*, *TLR21*, *RBL*, *HLEC*; Table 4 and Supplementary Material
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403 2, SM2), which indicate the immediate and prolonged activation of innate immune defense
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404 following NSAIDs exposure. Cellular biomarkers related to lipid metabolism were slightly affected
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405 by KET after 30 days and the significant enrichment of the biological process “fatty acid metabolic
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406 process”, the transcriptomic changes on *hydroxyacyl-Coenzyme A dehydrogenase*, *Acyl-Coenzyme*
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407 *A thioesterase* and *acetyl-Coenzyme A acyltransferase* corroborated the putative involvement of this
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408 pathway.

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409 Substantial differences were also observed in the transcriptional profile of genes related to DNA
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410 damage such as *DNA-damage inducible protein*, *nucleotide excision repair homolog*, *UV excision*
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411 *repair protein*, *growth arrest and DNA-damage-inducible alpha GADD45A*. *GADD45A*, here
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412 represented by two probes up-regulated at 30 days, is member of a group of nuclear proteins, whose
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413 transcript levels are increased following stressful growth arrest conditions and treatment with DNA-
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414 damaging agents. In detail, *GADD45A*, previously found to be up-regulated also in Manila clam
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415 exposed to ibuprofen (Milan et al. 2013), represents an important cell cycle checkpoint protein able
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416 to arrest cells at the G2/M phase. Quite interestingly, a unique probe representing a transcript
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417 coding for putative *cAMP responsive element binding protein-like 2 (CREBL2)* was revealed to be
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418 significantly down-regulated in all exposure times with fold change ranging between 8 and 21. This
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419 transcript is involved in adipose cells differentiation and play a regulatory role in the cell cycle
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420 which appeared to be modulated by KET also through the significant transcriptional changes of
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421 *Cyclin-I*, *Cell division cycle protein 123* and *cell division cycle 14 homolog A* at 14 days.

422 Apoptosis was found enriched at 14 and 60 days, with altered transcription of several genes playing
44
423 key role in apoptosis regulation, such as *CASP3*, *BIRC2*, *BIRC7*, *XIAP* and *BCL-2*. Despite
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424 knowledge is still limited on the molecular functions of these proteins in bivalve species, the
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425 obtained results confirmed the direct effects of NSAIDs on apoptosis regulation as previously
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426 shown in *M. galloprovincialis* and *R. philippinarum*, thus strengthening the hypothesis of
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427 similarities with signaling pathways identified in other model organisms (Chan et al. 2002; Din et
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428 al. 2005; Gu et al. 2005; Yin et al. 1998).

429 In conclusion this study demonstrated the ecotoxicological potential of NSAIDs in long-
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430 term exposure conditions. Beside the capability of mussels to constantly accumulate and/or
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431 metabolize anti-inflammatory drugs over 60 days, biological effects measured at both molecular
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6 and cellular level confirmed the immune system, lipid metabolism and cellular turnover as the main
732 biological targets of NSAIDs in mussels even in the absence of enhanced tissue concentrations.
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433 Future investigations will be needed to elucidate the effects of mixtures of NSAIDs with other
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434 contaminants, and the potential transfer of such compounds along the trophic chain.
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437 **5. Data accessibility**

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438 Gene expression analyses were performed using a 8 x 60 K Agilent oligo-DNA microarray platform
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439 deposited in the GEO database under accession number GPL18667
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440 (<http://www.ncbi.nlm.nih.gov/geo/>). Microarray raw and normalised fluorescence values were
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28
441 deposited in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number
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442 GSE103295.
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1 **Long-term exposure of *Mytilus galloprovincialis* to Diclofenac, Ibuprofen and Ketoprofen:**
2 **insights into bioavailability, biomarkers and transcriptomic changes.**

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26 **Abstract**

27 Non-steroidal anti-inflammatory drugs (NSAIDs) represent a growing concern for marine
28 ecosystems due to their ubiquitous occurrence and documented adverse effects on non-target
29 organisms. Despite the remarkable efforts to elucidate bioaccumulation and ecotoxicological
30 potential under short-term conditions, limited and fragmentary information is available for chronic
31 exposures. In this study bioavailability, molecular and cellular effects of diclofenac (DIC),
32 ibuprofen (IBU) and ketoprofen (KET) were investigated in mussels *Mytilus galloprovincialis*
33 exposed to the realistic environmental concentration of 2.5µg/L for up to 60 days. Results indicated
34 a significant accumulation of DIC and IBU but without a clear time-dependent trend; on the other
35 hand, KET concentrations were always below the detection limit. Analyses of a large panel of
36 molecular, biochemical and cellular biomarkers highlighted that all investigated NSAIDs caused
37 alterations of immunological parameters, genotoxic effects, modulation of lipid metabolism and
38 changes in cellular turn-over. This study provided the evidence of long-term ecotoxicological
39 potential of NSAIDs, further unraveling the possible hazard for wild marine organisms.

40 **Keywords:** Pharmaceuticals; Non Steroidal Anti-Inflammatory Drugs; marine mussels; long term
41 exposure; bioaccumulation; biomarkers; DNA-microarray.

42 **1. Introduction**

43 Over the last few years, the presence of pharmaceuticals in aquatic ecosystems has been recognized
44 as a crucial environmental issue. Occurrence in coastal areas was originally neglected, given the
45 high dilution capacity of seawater, but recent studies demonstrated the presence of such compounds
46 with concentrations ranging from a few ng/L to hundreds of µg/L (Fabbri and Franzellitti, 2015;
47 Lolić et al., 2015; Mezzelani et al., 2016). Adverse outcomes for marine organisms have been
48 mostly investigated through standard tests or laboratory exposures in short-term conditions,
49 typically from a few hours to 14 days (Aguirre-Martínez et al., 2015; Gonzales-Rey and Bebianno.,
50 2011, 2012, 2014; Mezzelani et al., 2016a, b; Milan et al., 2013; Trombini et al., 2016). Although
51 essential to establish acute toxicity, these data should be integrated with longer exposure studies to
52 characterize the possible occurrence of chronic effects, with prognostic and diagnostic value on the
53 impact of these ubiquitous molecules in marine biota (Cruz et al., 2016).

54 Among the most commonly used pharmaceuticals, non-steroidal anti-inflammatory drugs
55 (NSAIDs) represent a proper example of a therapeutic class with worldwide environmental
56 relevance: frequently measured in coastal areas (Lolić et al., 2015), they can be accumulated and
57 interfere with cellular pathways in marine organisms (Bebianno and Gonzalez-Rey, 2015;
58 Mezzelani et al., 2016a,b). In previous studies, the Mediterranean mussel *Mytilus galloprovincialis*
59 was exposed for 14 days at environmentally realistic concentrations of different NSAIDs (0.5-
60 25µg/L): these organisms demonstrated the capability to accumulate Diclofenac (DIC), Ibuprofen
61 (IBU) and Nimesulide (NIM) with a dose-dependent response, while Acetaminophen (AMP) and
62 Ketoprofen (KET) were never detectable in mussels tissues (Mezzelani et al., 2016a,b). All
63 NSAIDs, however, determined the onset of biochemical and cellular alterations with effects of
64 greater magnitude at the higher doses of exposure: immunological functions, lipid metabolisms and
65 cellular turnover appeared as the primarily modulated responses. These effects were confirmed at
66 molecular level for mussels exposed to 0.5 µg/L of KET and NIM, with significant changes of

67 transcriptional profile and expression of numbers of genes involved in eicosanoids metabolism,
68 immune system, apoptosis, cell-cycle regulation, reproduction and energetic metabolism (Mezzelani
69 et al., 2016b). Biochemical and cellular effects of DIC and IBU in *M. galloprovincialis* were
70 demonstrated also at a lower exposure concentration (0.25 µg/L), with a transitory induction of
71 antioxidant enzymes and increase of lipid peroxidation after 14-days (Gonzales-Rey and Bebianno.,
72 2011, 2012, 2014).

73 In addition to laboratory experiments, recent studies documented the presence of anti-inflammatory
74 drugs in tissues of wild marine organisms sampled along Italian and Spanish coasts (Mezzelani et
75 al., 2016a; Moreno-González et al., 2016). These evidences corroborate the hypothesis that aquatic
76 organisms are challenged by continuous release of pharmaceuticals in natural ecosystems raising
77 the need to elucidate whether chronic exposure conditions further exacerbate molecular,
78 biochemical and cellular outcomes in non target species (Peters and Granek, 2016).

79 In this respect, the present study investigated the long-term ecotoxicological potential of
80 DIC, IBU and KET in *M. galloprovincialis* exposed for up to 60 days to 2.5 µg/L, an
81 environmentally realistic level, similar to concentrations possibly occurring in coastal areas
82 (Castiglioni et al., 2005; Loos et al., 2013; Togola and Budzinski, 2008; Zuccato et al., 2006). The
83 work was based on a multidisciplinary approach integrating measurement of drug bioaccumulation
84 with a large number of biomarkers reflecting the perturbation of different cellular districts and
85 molecular pathways. Such responses included alteration of immunological parameters, lipid and
86 oxidative metabolism, onset of genotoxic effects. Considering the urgency of prioritize active
87 pharmaceuticals discharged in aquatic environment (Boxall et al., 2012), the biological relevance of
88 observed variations has been elaborated through a quantitative model and weighted criteria
89 (SediquaSoft) which summarize a cellular hazard index based on the toxicological importance of
90 analyzed biomarkers, number and magnitude of measured effects (Benedetti et al., 2012; Piva et al.,
91 2011).

92 Gene transcriptional profile was further analyzed in KET exposed mussels since this molecule was
93 already shown to cause significant effects after 14 days of treatment, independently on the lack of
94 bioaccumulation (Mezzelani et al., 2016b). In this respect, an additional objective of this study was
95 to investigate whether changes in gene expression can be considered as suitable biomarkers of
96 NSAIDs exposure also in long-term conditions, or if they rather represent transitory responses
97 before more integrated effects occur at cellular level.

98 The overall results of this study were expected to provide new insights on the potential risk of
99 constant exposure to NSAIDs in marine organisms contributing to elucidate links between cellular
100 effects and ecological consequences of pharmaceuticals in coastal areas.

101

102 **2. Materials and methods**

103 2.1 Experimental design

104 Stock solutions of Diclofenac, Ibuprofen and Ketoprofen (Sigma Aldrich) were stored at room
105 temperature (10 mg/L in methanol) and diluted in seawater during exposures of mussels.

106 Control organisms, *M. galloprovincialis* (5 ± 1 cm shell length), obtained from an unpolluted area
107 of Central Adriatic Sea (Portonovo, Ancona), were maintained for 10 days at constant laboratory
108 conditions (aerated seawater, $18 \pm 1^\circ\text{C}$, salinity 37, pH 8.0 ± 0.5 and oxygen saturation $>94\%$).

109 Mussels were randomly sub-divided in twelve 20 L aquarium (60 mussels per tank, for a total of
110 720 specimens). The experimental design included nine tanks with organisms exposed to $2.5 \mu\text{g/L}$
111 of DIC, IBU and KET respectively (3 tanks for each treatment), and three solvent control tanks
112 (CTRL) where methanol was added at the same concentration used in NSAIDs treatments
113 (0.003%). The water in each tank was daily changed and concentrations of various drugs were re-
114 established afterwards. Before water change, mussels were supplied with food (Zooplanktös-s™,
115 50-300 μm), and no mortality was observed during the experiment. At days 14, 30 and 60, whole
116 tissues were removed from 30 specimens for treatment (10 from each tank), pooled in 5 samples
117 (each constituted by tissues of 6 organisms) and stored at -20°C until chemical analyses. Moreover,

118 haemolymph, digestive glands and gills were immediately dissected from the remaining 30
119 specimens: 10 pools (each with tissues of 3 mussels) were frozen in liquid nitrogen and stored at -
120 80°C until biochemical and histochemical analyses. Lysosomal membrane destabilization,
121 phagocytosis and loss of DNA integrity (Comet assay) were immediately processed in haemocytes
122 using an aliquot of fresh haemolymph; another aliquot of chemically fixed haemolymph (Carnoy's
123 solution, 3:1 methanol, acetic acid) was microscopically analyzed for granulocytes-hyalinocytes
124 ratio and micronuclei frequency.

125 Samples for DNA microarray analysis were also prepared from CTRL and KET exposed mussels
126 (four pools, each with digestive glands of three organisms).

127

128 2.2 Chemical analyses

129 Protocols for measuring tissues concentrations of NSAIDs in exposed mussel by high performance
130 liquid chromatography, HPLC, were recently validated (Mezzelani et al., 2016a). For each group,
131 the five prepared pools were homogenized (1:1.6 w:v) in acetic acid 0.1%, pH=3.26 (buffer 1) at
132 room temperature for 20 minutes, and then centrifuged at 4,500 xg for 30 minutes. Samples were
133 purified by Solid Phase Extraction (SPE), using tubes pre-conditioned with methanol, ultra-pure
134 water and buffer, and recovering analytes with acetonitrile. After filtration and concentration by
135 SpeedVac, obtained samples were centrifuged again at 12,000 xg for 20 minutes.

136 Separations of KET and DIC were performed under isocratic condition with a mobile phase
137 composed by ultra-pure water (26%), acetonitrile (42%) and Buffer 1 (32%), elaborating
138 chromatographic signals by diode array detector (DAD) at 250 nm and 276 nm, for KET and DIC
139 respectively. IBU was separated on a gradient using ultra-pure water, acetonitrile and buffer 1 (from
140 35%:30%:35% to 0%:65%:35% for 23 minutes), and detected by fluorimetric detector (FD) with
141 excitation/emission wavelengths at 230/294 nm. Pure standard solutions of various NSAIDs were
142 used to quantify concentrations measured in mussels' tissues. Considering the absence of Standard
143 Reference Materials (SRMs), a series of blank samples were spiked with various doses of pure

144 NSAIDs standards to assess the reproducibility, quality assurance and quality control of analytical
145 procedures (Mezzelani et al., 2016a). The recovery yield was always $\geq 98\%$ ($CV < 5\%$, $n=10$), with
146 an instrumental limit of detection (LOD) in mussel tissues of 1 ng/g dry weight (d.w.) for DIC and
147 0.5 ng/g (d.w.) for IBU and KET.

148

149 2.3 Biological responses

150 Validated protocols were used to analyze the following biomarkers in tissues of exposed mussels:
151 immunological responses as lysosomal membrane stability (neutral red retention time, NRRT),
152 granulocytes/hyalinocytes ratio (G/H) and phagocytosis activity in haemocytes; peroxisomal
153 proliferation (Acyl-CoA oxidase, ACOX) and neurotoxic effects (acetylcholinesterase, AChE) in
154 haemolymph and gills; antioxidants defences and susceptibility to oxidative stress in digestive
155 gland (catalase CAT, glutathione S-transferases GST, Se-dependent glutathione peroxidases GPx
156 H₂O₂, total GPx CHP, glutathione reductase GR, total glutathione GSH, total antioxidant capacity
157 TOSC toward peroxy radicals ROO• and hydroxyl radicals HO•); accumulation of lipid
158 peroxidation products (lipofuscin, LIPO) and neutral lipids (NL) in digestive gland; loss of DNA
159 integrity (DNA strand breaks and micronuclei, MN) in haemocytes. Detailed protocols are given in
160 Supplementary Material 1 (SM1).

161

162 2.4 Labelling, microarray hybridization and data acquisition

163 Transcriptional profile was analyzed using an 8x60K Agilent oligo-DNA microarray in CTRL and
164 KET groups, using 4 pools of digestive glands, each containing tissues of 3 individuals. This
165 platform, developed within the REPROSEED European project (REsearch project to improve
166 PROduction of SEED of established and emerging bivalve species in European hatcheries) contains
167 59,997 probes, representing a total of 50,680 contigs for *M. galloprovincialis*. Details on the
168 microarray and probe sequences are given in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>,
169 accession number GPL18667) while other detail on sequencing, assembly, annotation and

170 microarray design are summarized in Supplementary Material 1 (SM1). Microarrays were
171 synthesized through the *in situ* non-contact ink-jet technology including default positive and
172 negative controls, and to the Agilent One-Color Microarray-Based Gene Expression Analysis
173 protocol was used for sample labelling and hybridization with the Low Input Quick Amp Labeling
174 kit. Labelling, hybridization and data acquisition procedures are detailed in Supplementary Material
175 2 (SM2).

176 Normalization procedures included quantile normalization, which always outperformed cyclic
177 loess. Data on raw gene expression and normalized data can be accessed in the GEO database
178 (GSE103295 and GSE103295, respectively).

179

180 2.5 Statistical analyses

181 Analysis of variance was applied to test differences between treatments and exposure days (level of
182 significance at $p < 0.001$). Cochran C was applied for testing homogeneity of variance, with
183 appropriate mathematical transformation if necessary; the Student Newman-Keuls test, (SNK) was
184 used for post-hoc comparison between means of values ($n=5$). Statistical analyses were performed
185 using R-cran software (<http://www.R-project.org>).

186 The overall biological significance of biomarker responses was summarized in a hazard index with
187 a quantitative, software-assisted model (Sediqualsoft), which uses weighted criteria to elaborate
188 large data-sets of heterogeneous data (Benedetti et al., 2012, 2014; Piva et al., 2011). Briefly, all
189 biomarkers have a specific “weight”, depending on the toxicological relevance of the endpoint, and
190 a “threshold” which defines the minimum percentage of variation considered of biological
191 significance. Each biomarker response is compared to the respective threshold, corrected for both
192 the statistical significance of the variation and the toxicological weight of the response: detailed
193 flow-charts, rationale for weights, thresholds and expert judgements have been fully given
194 elsewhere (Benedetti et al., 2012, 2014; Piva et al., 2011). The Hazard Quotient for biomarker
195 variations (HQ_{BM}) is calculated without considering the Effects lower or equal to respective

196 thresholds, averaging those with an effect up to two-fold compared to the threshold, and adding the
197 summation (Σ) for the biomarker responses more than 2 fold greater than the respective threshold
198 (Benedetti et al., 2012, 2014; Piva et al., 2011):

199

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

200

201

202 The calculated HQ_{BM} is finally attributed to one of five classes of cumulative hazard for biomarkers
203 (from Absent to Severe), maintaining scientifically sound information, while also being easy to read
204 for non-expert stakeholders (Benedetti et al., 2012, 2014; Piva et al., 2011).

205 Transcriptional results were statistical analyzed selecting 45,502 out of 59,971 probes with signal
206 higher than the background in at least 4 out of 24 analysed samples. Principal Component Analysis
207 (PCA) and T-test statistics (Bonferroni correction for multiple comparisons; p-value<0.05; 200
208 permutations) elaborated results obtained from CTRL and KET exposed mussels at each time, 14,
209 30 and 60 days (TIGR Multi Experiment Viewer, TMeV 4.5.1 statistical software, by Saeed et al.,
210 2003).

211 Only probes with fold change (FC) > 1.5 and signal higher than the background in at least one
212 group have been considered as differentially expressed genes (DEGs). Functional connections of
213 observed DEGs were investigated using Database for Annotation, Visualization, and Integrated
214 Discovery (DAVID) software (Huang et al., 2009), matching transcripts of *M. galloprovincialis* to
215 *Danio rerio* Gene IDs using dedicated Blast searches performed with blastx (E-value < 10⁻⁵). Since
216 functional annotation data are available for a limited number of species, the choice of *D. rerio* was
217 previously demonstrated to allow the assignment of a putative homologue to a larger number of
218 transcripts in bivalves, such as *M. galloprovincialis* (Avio et al., 2015), and *Ruditapes*

219 *philippinarum* (Milan et al., 2011, 2013). Genes differentially expressed in each T-test pairwise
220 comparison, were functionally annotated setting DAVID for gene count=2 and ease=0.1.
221

222 3. Results

223 Levels of NSAIDs in control and exposed mussels are reported in Table 1. While values were
224 always below the instrumental limit of detection (LOD) in controls, an increase of tissue
225 concentrations was observed in DIC and, particularly, in IBU-exposed organisms. In both the
226 treatments, values measured after 14 days did not statistically change over the following exposure
227 period, despite mean values of IBU in mussels tissues almost doubled at day 60 compared to
228 previous times. Tissue levels of KET were always below LOD.

229 Results of analyzed biomarkers are reported in Table 2, while those of two-way analysis of
230 variance (ANOVA) are given in Table 1 of Supplementary Material 1 (SM1). To facilitate the
231 comparison between the effects caused by different NSAIDs at various days of exposure,
232 biomarkers with statistically significant changes are also shown as percentage variations compared
233 to relative control organisms (Figure 1).

234 Immunological responses were evident as reduction of lysosomal membrane stability in mussels
235 exposed to IBU and KET after 14, 30 and 60 days; the same trend, although not statistically
236 significant, was observed for DIC treatment (Fig. 1A). The granulocytes/hyalinocytes ratio was
237 decreased by all tested NSAIDs (Fig. 1B): time dependent effects were measured for DIC and IBU,
238 while the sharp reduction caused by KET after 14 days remained almost constant during the
239 following exposure period. Phagocytosis capacity exhibited an inhibition trend for all treatments
240 that, however, resulted statistically significant only for IBU and KET after 30 days of exposure
241 (Fig. 1C).

242 Lipofuscin content in tertiary lysosomes appeared markedly enhanced only in organisms exposed to
243 KET after 60 days. (Fig. 1D), while neutral lipids significantly increased after 30 days in organisms
244 treated with IBU and KET (Fig. 1E). The activity of acyl CoA oxidase tended to be decreased by
245 various NSAIDs with a significant effect in KET-exposed organisms after 30 days (Fig. 1F).

246 The measurement of genotoxic effects revealed a generalized increase of DNA strand breaks with
247 significantly higher values in mussels exposed to DIC for 14 and 60 days and to KET for 14 days

248 (Fig. 1G). Levels of micronuclei exhibited a marked, but transitory enhancement for all the
249 treatments, specifically after 30 days for DIC, after 14 and 30 days for IBU and KET (Fig. 1H).

250 No significant variations were measured for acetylcholinesterase in both haemolymph and gills at
251 various treatments and exposure period, and antioxidant defenses (catalase, glutathione reductase,
252 glutathione peroxidases, glutathione S-transferases and levels of glutathione) also showed only
253 limited and not statistically significant variations between the different experimental conditions
254 (Table 2). The lack of prooxidant effects induced by anti-inflammatory pharmaceuticals in mussels
255 was further supported by the results on Total Oxyradical Scavenging Capacity with almost constant
256 values toward both peroxy and hydroxyl radicals (Table 2).

257 Considering the biological relevance (weight) and magnitude of observed effects, the elaboration of
258 biomarker results with weighted criteria summarized as respectively “Slight” and “Moderate” the
259 hazard for organisms exposed to DIC and IBU over the entire exposure period. In KET exposed
260 organisms, the level “Slight” obtained after 14 days increased to “Moderate” after 30 and 60 days of
261 treatment (Table 3).

262 Transcriptional changes in KET exposed mussels, highlighted a total of 674, 469 and 447
263 differentially expressed genes (DEGs) at 14, 30 and 60 days, respectively (Figure 2); the full list of
264 DEGs is reported in Supplementary Material 2 (SM2). The Venn diagram revealed that a unique
265 probe, coding for putative cAMP responsive element binding protein-like 2 (CREBL2), was
266 significantly down-regulated at all times of exposure (Fig.2). Other DEGs included transcripts
267 involved in the arachidonic acid (AA) metabolism (*phospholipase A2 PLA2*, *arachidonate 5-*
268 *lipoygenase ALOX5*), immune responses (*toll-like receptors TLR2, TLR4, TLR4B, TLR 7, TLR21,*
269 *Rhamnose-binding lectin RBL, hepatic lectin HLEC*), lipid metabolism (*hydroxyacyl-Coenzyme A*
270 *dehydrogenase ACOT13, Acyl-Coenzyme A thioesterase HADH, acetyl-Coenzyme A acyltransferase*
271 *1 ACAA1*), cell cycle (*Cyclin-I CCN1, Cell division cycle protein 123 CDC 123, cell division cycle*
272 *14 homolog A CDC14*), apoptosis (*baculoviral IAP repeat-containing 2 BIRC2, baculoviral IAP*
273 *repeat-containing 7 BIRC7, X-linked inhibitor of apoptosis XIAP, caspase 3 CASP3, DNA-damage*

274 *regulated autophagy modulator 2a DRAM2, DNA fragmentation factor subunit beta DFFB*) and
275 DNA damage (*DNA-damage inducible protein DDIT1, nucleotide excision repair homolog MMS19,*
276 *UV excision repair protein RAD23, growth arrest and DNA-damage-inducible alpha GADD45A*).
277 The enrichment analysis with DAVID software (Dennis et al. 2003; Huang et al. 2009) provided a
278 more systematic functional interpretation of observed DEGs. This analysis has been performed
279 separately for down- and up-regulated genes in response to KET exposure. Lists of significantly
280 enriched Biological Process (BP), Cellular Component (CC), Molecular Function (MF) GO terms
281 and KEGG pathways are fully reported in Supplementary Material 3 (SM3), and summarized in
282 Table 4. The most represented enriched terms included “endocytosis” and “oxidation reduction” (at
283 14 and 30 days), “apoptosis” (at 14 and 60 days), “RNA processing” (at 14 days), “macromolecule
284 catabolic process”, “NOD-like receptor signaling pathway” (at 30 and 60 days), “fatty acid
285 metabolic and biosynthetic process” (at 30 days), and “Toll-like receptor signaling pathway” (at 60
286 days).

287

288 **4. Discussion**

289 The present investigation aimed to study the long-term ecotoxicological potential of NSAIDs in *M.*
290 *galloprovincialis*, providing novel insights on possible adverse effects and hazard in coastal areas.
291 Pharmaceuticals should be considered as high priority environmental pollutants (Aguirre-Martínez
292 et al., 2016; Bebianno and Gonzalez-Rey, 2015; Daughton, 2016; Fabbri, 2015; He et al., 2017) and
293 a particular concern has been raised for NSAIDs, one of the most consumed therapeutical class
294 worldwide, with ubiquitous occurrence in aquatic ecosystems (Brune and Patrignani, 2015; He et
295 al., 2017; Kim et al., 2015; Paíga et al., 2013). Such compounds were elected as ideal candidates for
296 eco-pharmacoavilance investigations aimed to characterize the distribution, adverse effects and
297 fate of pharmaceuticals in the environment to implement knowledge-based reduction and
298 prevention measures (He et al., 2017; Holm et al., 2013; Nieto et al., 2017). In this respect, physic,
299 chemical and biological properties of NSAIDs should all be considered when assessing their

300 environmental impact (McEneff et al., 2014). In fact, although susceptible to degradation and with a
301 half-life usually shorter than other pollutants, the continuous introduction into the aquatic
302 environment actually confers to NSAIDs pseudo-persistence characteristics (Ankley et al., 2007;
303 Hernando et al., 2006). In our study, mussels exposed to the environmentally realistic concentration
304 of 2.5µg/L for 60 days, showed measurable levels of DIC and IBU, after 14 days without a clear
305 time-dependent increase at longer exposure periods: a similar result may suggest the activation of
306 detoxification pathways or excretion processes leading to a steady state of NSAIDs tissue
307 concentrations. These results confirmed the possibility of marine mussels to accumulate
308 pharmaceuticals over a wide range of environmental levels: the comparison of tissue levels
309 measured in this study with those observed in organisms treated with 0.5 or 25µg/L of the same
310 compounds, highlight a possible dose dependent behavior for DIC, but not for IBU (Mezzelani et
311 al., 2016a,b), underling that molecules with similar mechanisms of action do not have the same
312 pharmacokinetic pathways. This aspect is further corroborated by the lack of accumulation of KET,
313 always below the LOD, independently from the exposure times and concentrations used in the
314 present or in the previous investigations (Mezzelani et al., 2016a,b). Considering the onset of
315 biological effects in organisms exposed to this drug, it has already been suggested that the lack of
316 KET accumulation in mussels tissues probably reflect the transformation of this molecule either in
317 the environment or within cells once accumulated. Worthy to note, levels of anti-inflammatory
318 drugs measured in laboratory experiments are comparable with those measured in wild mussels
319 from the Italian coasts (Mezzelani et al., 2016a) with similar concentrations for DIC and IBU, while
320 never detectable for KET.

321 Among the wide panel of analyzed biological responses, lysosomal membrane stability was
322 confirmed as a sensitive biomarker for detecting adverse effects of anti-inflammatory compounds,
323 with an early responsiveness at 14 days, then constantly maintained over 60 days of exposure. The
324 comparison with previous experiments performed in a wider range of environmentally realistic
325 concentrations (0.5-2.5-25µg/L) (Mezzelani et al., 2016a,b) reveal a dose dependent modulation of

326 this parameter for DIC and KET, while IBU appeared to strongly reduce lysosomal stability at all
327 the doses of exposure. This study further supported the modulation of immune system with the
328 significant decrease of granulocytes/hyalinocytes ratio and the parallel inhibition of phagocytosis
329 capacity: the latter effect is presumably related to the lower phagocytic capacity of hyalinocytes
330 compared to granulocytes which normally represent the dominant cell type in haemolymph of *M.*
331 *galloprovincialis* (Gorbi et al., 2012, 2013), responsible for cell-mediated immunity through
332 phagocytosis and cytotoxic reactions (Ciacci et al., 2012). Despite precise mechanisms behind the
333 modulation of these pathways remain unclear, the present study unravels the suitability of immune
334 system biomarkers as targets of long-term exposure to anti-inflammatory compounds. Antioxidant
335 biomarkers generally showed quite constant values of catalase, glutathione dependent enzymes and
336 level of total glutathione. The lack of oxidative challenge determined by NSAIDs was further
337 confirmed by the total oxyradical scavenging capacity toward peroxy and hydroxyl radicals which
338 were never affected by any experimental condition. These findings, in agreement with previous
339 short-term investigations on marine bivalves, confirm that prooxidant mechanisms do not represent
340 the primary mode of action of these pharmaceuticals neither in short, nor in long-term exposure
341 conditions (Bebiano and Gonzalez-Rey, 2015; Bebianno et al., 2017; Mezzelani et al., 2016 a, b).
342 The limited oxidative challenge of NSAIDs was reflected by results obtained on lipofuscin, an
343 intralysosomal non degradable product, primarily composed of cross-linked proteins and lipids
344 accumulated under oxidative stress conditions (Regoli, 1992). An appreciable increment was
345 measured in KET exposed mussels only at the end of the treatment while a more generalized
346 accumulation of lipofuscin had been previously documented after 14 days of exposure to higher
347 levels of NSAIDs (Mezzelani et al., 2016a,b). Variable and fluctuating results on lipofuscin content
348 are quite common in marine organisms exposed to active pharmaceutical ingredients (Gonzalez-
349 Rey et al., 2014; Nunes et al., 2017): unless challenged by prooxidant chemicals, lipofuscin can be
350 modulated by various biological factors including reproductive cycle, feeding activities and
351 autophagic processes to cite a few. In this study, a transient increase of neutral lipids was observed

352 in mussels exposed to NSAIDs, contrasting with recent investigations showing a high degradation
353 of such energy reserves, possibly related to a direct modulation of lipid metabolism mediated by the
354 drugs (Mezzelani et al., 2016a,b). However, it should be noted that control organisms had
355 particularly low basal levels of neutral lipids which reflected the pronounced seasonal pattern of
356 these reserves with minimum levels during autumn and winter season when glycogen is mostly
357 utilized (Regoli et al., 1992). In this respect, the present study revealed slight changes also in the
358 activity of the Acyl CoA oxidase (ACOX) which catalyzes the first reaction of β -mitochondrial
359 oxidation of fatty acids revealing a potential change in metabolisms of energy resources, only in
360 KET exposed mussels. Long-term exposure to anti-inflammatory drugs determined limited
361 fluctuations on the AchE activity, which has not been highlighted as a main target of NSAIDs also
362 in previous investigations on mussels, showing a moderate induction in gills after 3 and 7 days of
363 exposure to 250 ng/L of DIC (Gonzalez-Rey and Bebianno, 2014, Mezzelani et al., 2016a,b).
364 Genotoxic damages expressed as DNA fragmentation and frequency of micronuclei tended to
365 increase in mussels response to all investigated compounds with time dependent enhancement of
366 DNA strand breaks for DIC and IBU exposed mussels. The frequency of micronuclei over 60 days
367 of exposure showed a typical biphasic trend for all investigated drugs, corroborating the hypothesis
368 that NSAIDs would not increase MN acting as direct genotoxic compounds on DNA integrity, but
369 rather influencing the haemocytes cellular turn-over (Chang et al., 2009; Mezzelani et al., 2016a,b).
370 The elaboration of all biomarkers data within the Weight Of Evidence model suggest that biological
371 responses quickly reach their steady state conditions in mussels exposed to DIC and IBU where the
372 summarized hazard indices were “Slight” and “Moderate” after 14 days, then remaining constant
373 for the entire duration of exposure; only in KET exposed organisms the hazard level elaborated as
374 “Slight” after 14 days increased to “Moderate” at 30 and 60 days of treatment. These results
375 confirm that, despite concentrations of KET in mussels tissues were always below the LOD,
376 biological effects are evident and tend to increase in chronic conditions, reinforcing the hypothesis
377 on bioavailability and potential (bio)-transformation of this drug (Mezzelani et al., 2016a,b).

378 To further corroborate this possibility, transcriptomic profile was analyzed in KET exposed
379 mussels. Combining cellular biomarkers responses with transcriptomic analysis is of particular
380 relevance for emerging contaminants, to obtain new insights on their mechanisms of action (MOA).
381 Since aquatic wildlife may possess conserved targets related to the drug metabolism,
382 pharmaceuticals are potentially able to alter essential homeostatic mechanisms even at
383 concentrations lower than those for human dosage (Fabbri and Franzellitti, 2015; Maria et al.,
384 2016).

385 Overall, the number of DEGs was more elevated after 14 days (674) compared to 30 and 60 days
386 (469 and 477 DEGs, respectively), which may suggest that cellular response is initially
387 characterized by a generalized early modulation of several pathways while, at more prolonged
388 exposure, a higher specificity of DEGs would prevail. In mammals, NSAIDs inhibit the reaction
389 catalyzed by the cyclo-oxygenase enzymes COX-1 and COX-2, preventing the formation of
390 prostaglandins and thromboxane from arachidonic acid (Gierse et al., 1995; Parolini et al., 2011). In
391 this work, transcriptional profiles provided evidence on the effects of KET toward arachidonic acid
392 (AA) metabolism in mussels. PLA2 is a key enzyme in catalyzing the hydrolysis of cellular
393 phospholipids at the sn-2 position to liberate AA during eicosanoids metabolism; its up-regulation
394 after 60 days of KET exposure may indicate a potential recruitment of AA to increase the substrate
395 availability as a result of COX inhibition. This finding confirmed the results obtained in mussels
396 exposed to 0.5µg/L of KET (Mezzelani et al., 2016 b) and in Manila clam exposed to IBU (Milan et
397 al. 2011), further highlighting direct ecotoxicological effects of NSAIDs on AA metabolism with
398 potentially deleterious consequences in reproduction, gametogenesis and larval settlement (Knight
399 et al., 2000; Stanley, 2000).

400 Molecular analyses strongly supported variations observed for cellular biomarkers, in particular
401 those on immune responses with changes in a large number of genes regulating toll-like receptors,
402 and lectins (*TLR2*, *TLR4*, *TLR4B*, *TLR7*, *TLR21*, *RBL*, *HLEC*; Table 4 and Supplementary Material
403 2, SM2), which indicate the immediate and prolonged activation of innate immune defense

404 following NSAIDs exposure. Cellular biomarkers related to lipid metabolism were slightly affected
405 by KET after 30 days and the significant enrichment of the biological process “fatty acid metabolic
406 process”, the transcriptomic changes on *hydroxyacyl-Coenzyme A dehydrogenase*, *Acyl-Coenzyme*
407 *A thioesterase* and *acetyl-Coenzyme A acyltransferase* corroborated the putative involvement of this
408 pathway.

409 Substantial differences were also observed in the transcriptional profile of genes related to DNA
410 damage such as *DNA-damage inducible protein*, *nucleotide excision repair homolog*, *UV excision*
411 *repair protein*, *growth arrest and DNA-damage-inducible alpha* *GADD45A*. *GADD45A*, here
412 represented by two probes up-regulated at 30 days, is member of a group of nuclear proteins, whose
413 transcript levels are increased following stressful growth arrest conditions and treatment with DNA-
414 damaging agents. In detail, *GADD45A*, previously found to be up-regulated also in Manila clam
415 exposed to ibuprofen (Milan et al. 2013), represents an important cell cycle checkpoint protein able
416 to arrest cells at the G2/M phase. Quite interestingly, a unique probe representing a transcript
417 coding for putative *cAMP responsive element binding protein-like 2* (*CREBL2*) was revealed to be
418 significantly down-regulated in all exposure times with fold change ranging between 8 and 21. This
419 transcript is involved in adipose cells differentiation and play a regulatory role in the cell cycle
420 which appeared to be modulated by KET also through the significant transcriptional changes of
421 *Cyclin-I*, *Cell division cycle protein 123* and *cell division cycle 14 homolog A* at 14 days.

422 Apoptosis was found enriched at 14 and 60 days, with altered transcription of several genes playing
423 key role in apoptosis regulation, such as *CASP3*, *BIRC2*, *BIRC7*, *XIAP* and *BCL-2*. Despite
424 knowledge is still limited on the molecular functions of these proteins in bivalve species, the
425 obtained results confirmed the direct effects of NSAIDs on apoptosis regulation as previously
426 shown in *M. galloprovincialis* and *R. philippinarum*, thus strengthening the hypothesis of
427 similarities with signaling pathways identified in other model organisms (Chan et al. 2002; Din et
428 al. 2005; Gu et al. 2005; Yin et al. 1998).

429 In conclusion this study demonstrated the ecotoxicological potential of NSAIDs in long-
430 term exposure conditions. Beside the capability of mussels to constantly accumulate and/or
431 metabolize anti-inflammatory drugs over 60 days, biological effects measured at both molecular
432 and cellular level confirmed the immune system, lipid metabolism and cellular turnover as the main
433 biological targets of NSAIDs in mussels even in the absence of enhanced tissue concentrations.
434 Future investigations will be needed to elucidate the effects of mixtures of NSAIDs with other
435 contaminants, and the potential transfer of such compounds along the trophic chain.

436

437 **5. Data accessibility**

438 Gene expression analyses were performed using a 8 x 60 K Agilent oligo-DNA microarray platform
439 deposited in the GEO database under accession number GPL18667
440 (<http://www.ncbi.nlm.nih.gov/geo/>). Microarray raw and normalised fluorescence values were
441 deposited in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number
442 GSE103295.

443

444

445 **6. References**

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TABLES

Table 1. Bioaccumulation of DIC, IBU and KET in *M. galloprovincialis* exposed to 2.5 µg/L. Data are given as ng/g dry weight (mean values ± standard deviation, n = 5).

Compound	Treatment	Exposure time (days)		
		14	30	60
DIC	Control	<LOD	<LOD	<LOD
	Exposed	1.63 ± 1.56	3.63 ± 1.40	2.25 ± 0.810
IBU	Control	<LOD	<LOD	<LOD
	Exposed	25.6 ± 44.3	24.6 ± 16.6	45.1 ± 25.5
KET	Control	<LOD	<LOD	<LOD
	Exposed	<LOD	<LOD	<LOD

Table 2. Biomarker responses in *M. galloprovincialis* exposed to 2.5 µg/L of DIC, IBU and KET. Data are given as mean values ± standard deviation, n = 5.

Biomarker	Exposure time (days)	Treatment			
		CTRL	DIC	IBU	KET
Neutral Red Retention Time (min)	0	86.5 ± 8.28	86.5 ± 8.28	86.5 ± 8.28	86.5 ± 8.28
	14	80.9 ± 7.54	63.0 ± 12.8	34.5 ± 9.60	58.8 ± 4.89
	30	70.1 ± 7.64	48.8 ± 13.2	45.5 ± 7.30	39.0 ± 5.17
	60	50.1 ± 3.58	32.6 ± 10.0	28.1 ± 6.56	27.0 ± 8.50
Granulocytes/Hyalinocytes ratio	0	4.86 ± 0.972	4.86 ± 0.972	4.86 ± 0.972	4.86 ± 0.972
	14	5.40 ± 0.690	5.52 ± 1.56	3.05 ± 0.310	1.52 ± 0.440
	30	6.70 ± 1.01	3.32 ± 0.730	2.17 ± 0.250	2.59 ± 0.520
	60	6.25 ± 1.92	0.880 ± 0.200	1.54 ± 0.530	1.74 ± 0.550
Phagocytosis capacity (%)	0	34.9 ± 2.31	34.9 ± 2.31	34.9 ± 2.31	34.9 ± 2.31
	14	34.8 ± 2.88	27.8 ± 13.4	39.3 ± 10.5	39.8 ± 9.23
	30	41.3 ± 11.3	37.7 ± 5.93	23.2 ± 10.3	13.8 ± 4.69
	60	44.2 ± 3.84	30.6 ± 5.32	23.1 ± 7.44	6.89 ± 4.61
Acetylcholinesterase haemolymph (nmol/min/mg prt)	0	106 ± 44.7	106 ± 44.7	106 ± 44.7	106 ± 44.7
	14	99.3 ± 36.0	112 ± 13.4	74.0 ± 33.8	95.1 ± 53.5
	30	153 ± 60.2	93.9 ± 27.0	93.5 ± 19.9	85.3 ± 18.0
	60	109 ± 34.2	89.6 ± 18.2	63.5 ± 8.85	64.0 ± 7.42
Acetylcholinesterase gills (nmol/min/mg prt)	0	9.93 ± 4.04	9.93 ± 4.04	9.93 ± 4.04	9.93 ± 4.04
	14	10.5 ± 3.21	11.3 ± 2.16	14.2 ± 3.38	11.2 ± 2.65
	30	8.02 ± 1.29	11.3 ± 3.39	9.91 ± 1.10	9.60 ± 2.96
	60	6.02 ± 1.19	10.5 ± 2.73	6.25 ± 1.42	5.35 ± 1.03
Catalase (µmol/min/mg prt)	0	17.2 ± 1.09	17.2 ± 1.09	17.2 ± 1.09	17.2 ± 1.09
	14	16.5 ± 2.40	15.4 ± 1.97	9.74 ± 1.36	17.7 ± 7.25
	30	11.2 ± 8.14	13.0 ± 8.15	15.5 ± 3.37	17.7 ± 8.40
	60	18.0 ± 4.80	15.3 ± 2.49	13.4 ± 5.11	13.4 ± 6.23
Glutathione S-transferases (nmol/min/mg prot)	0	80.8 ± 8.34	80.8 ± 8.34	80.8 ± 8.34	80.8 ± 8.34
	14	77.7 ± 8.63	86.3 ± 21.1	91.7 ± 14.9	100 ± 21.9
	30	93.1 ± 7.80	92.8 ± 10.3	95.4 ± 23.1	84.4 ± 5.78
	60	71.5 ± 8.60	94.0 ± 4.26	82.3 ± 6.06	76.0 ± 8.75
Glutathione peroxidases CHP (nmol/min/mg prot)	0	7.97 ± 1.45	7.97 ± 1.45	7.97 ± 1.45	7.97 ± 1.45
	14	8.13 ± 1.20	5.75 ± 3.32	7.22 ± 3.92	11.9 ± 2.09
	30	10.2 ± 6.48	10.2 ± 2.30	8.22 ± 1.00	8.93 ± 1.05
	60	10.5 ± 2.43	10.6 ± 3.94	7.27 ± 1.02	9.21 ± 1.28
Glutathione peroxidases H ₂ O ₂ (nmol/min/mg prot)	0	1.93 ± 0.926	1.93 ± 0.926	1.93 ± 0.926	1.93 ± 0.926
	14	1.94 ± 1.42	2.95 ± 0.397	1.56 ± 0.712	2.05 ± 0.053
	30	3.80 ± 3.83	3.00 ± 0.280	2.29 ± 1.24	3.02 ± 0.675
	60	2.89 ± 1.11	4.06 ± 1.78	2.38 ± 1.53	1.53 ± 0.209
Glutathione reductase (nmol/min/mg prot)	0	22.2 ± 4.48	22.2 ± 4.48	22.2 ± 4.48	22.2 ± 4.48
	14	23.6 ± 2.85	19.6 ± 2.51	20.2 ± 0.525	23.3 ± 3.20
	30	17.6 ± 5.78	16.6 ± 3.95	22.7 ± 6.03	19.1 ± 1.81
	60	16.6 ± 2.99	16.6 ± 0.52	20.5 ± 1.65	19.3 ± 1.96
Total glutathione (µmol/g tissue)	0	0.841 ± 0.258	0.841 ± 0.258	0.841 ± 0.258	0.841 ± 0.258
	14	0.845 ± 0.373	0.744 ± 0.060	0.628 ± 0.098	0.754 ± 0.089
	30	0.752 ± 0.193	0.565 ± 0.078	0.796 ± 0.144	0.637 ± 0.101
	60	0.545 ± 0.080	0.622 ± 0.203	0.683 ± 0.121	0.663 ± 0.150
Total Oxyradical Scavenging Capacity •OOR (Utosc/mg prot)	0	300 ± 26.8	300 ± 26.8	300 ± 26.8	300 ± 26.8
	14	304 ± 35.1	293 ± 19.1	322 ± 55.7	334 ± 40.2
	30	298 ± 67.8	388 ± 71.5	302 ± 46.7	396 ± 58.3
	60	406 ± 68.7	445 ± 22.5	439 ± 71.5	330 ± 40.7
Total Oxyradical Scavenging Capacity •OH (Utosc/mg prot)	0	270 ± 48.6	270 ± 48.6	270 ± 48.6	270 ± 48.6
	14	280 ± 55.0	348 ± 90.8	286 ± 18.0	262 ± 12.5
	30	340 ± 77.1	378 ± 141	332 ± 34.8	328 ± 47.6
	60	337 ± 63.5	377 ± 68.0	377 ± 67.4	316 ± 44.0
Lipofuscin (int/µm ²)	0	6.10 ± 0.815	6.10 ± 0.815	6.10 ± 0.815	6.10 ± 0.815
	14	6.57 ± 1.95	5.67 ± 1.87	5.55 ± 1.74	10.16 ± 2.31
	30	11.4 ± 9.76	18.0 ± 1.88	13.4 ± 5.89	13.5 ± 5.82
	60	3.67 ± 1.48	4.62 ± 0.240	6.62 ± 2.62	14.43 ± 1.45
Neutral Lipids (int/µm ²)	0	2.53 ± 0.372	2.53 ± 0.372	2.53 ± 0.372	2.53 ± 0.372
	14	2.43 ± 0.43	12.1 ± 15.9	4.10 ± 4.18	6.16 ± 3.55
	30	2.55 ± 0.620	2.91 ± 1.47	31.1 ± 14.4	13.5 ± 8.76
	60	2.67 ± 1.18	3.44 ± 4.17	2.73 ± 1.90	5.85 ± 4.54
Acyl-CoA oxidase (nmol/min/mg prt)	0	0.812 ± 0.158	0.812 ± 0.158	0.812 ± 0.158	0.812 ± 0.158
	14	0.706 ± 0.051	0.431 ± 0.246	0.802 ± 0.159	0.639 ± 0.229
	30	0.610 ± 0.130	0.490 ± 0.130	0.570 ± 0.130	0.290 ± 0.034
	60	0.427 ± 0.094	0.282 ± 0.022	0.294 ± 0.082	0.316 ± 0.103
DNA fragmentation (%)	0	11.1 ± 2.83	11.1 ± 2.83	11.1 ± 2.83	11.1 ± 2.83
	14	10.3 ± 3.55	16.9 ± 2.12	16.6 ± 1.87	20.5 ± 1.55
	30	27.0 ± 4.79	36.7 ± 4.21	39.4 ± 3.28	35.4 ± 2.87
	60	26.7 ± 1.81	57.0 ± 2.65	47.4 ± 9.14	33.9 ± 3.18
Micronuclei frequency (‰)	0	0.100 ± 0.100	0.100 ± 0.100	0.100 ± 0.100	0.100 ± 0.100
	14	0.100 ± 0.100	0.100 ± 0.100	1.09 ± 0.51	0.595 ± 0.101
	30	0.185 ± 0.185	3.49 ± 0.611	3.40 ± 1.03	1.12 ± 0.424
	60	1.61 ± 0.126	1.58 ± 0.476	1.62 ± 0.553	2.48 ± 0.628

Table 3. Class of hazard elaborated for biomarkers data in mussels exposed to different NSAIDs.

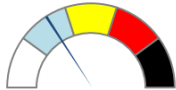

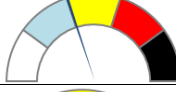






Compound	Day	Class of Hazard	Level
DIC	14	SLIGHT	
	30	SLIGHT	
	60	SLIGHT	
IBU	14	MODERATE	
	30	MODERATE	
	60	MODERATE	
KET	14	SLIGHT	
	30	MODERATE	
	60	MODERATE	

Table 4. Lists of the main biological processes and KEGG pathways modulated by KET at each time of exposure. The arrow ↑ indicates enriched BP and KEGG pathways considering up-regulated genes in response to ketoprofen, while ↓ indicates enriched BP and KEGG pathways considering down-regulated genes in response to ketoprofen. N°: number of differentially expressed genes for each enriched BP and KEGG pathways; FE: Fold enrichment.

Time of exposure	Category	Term	N°	p-value	FE
14 days	BP	↓ GO:0055114~oxidation reduction	16	0.00	2.30
	BP	↓ GO:0046777~protein amino acid autophosphorylation	2	0.09	20.8
	KEGG	↓ dre04144:Endocytosis	8	0.03	2.50
	KEGG	↓ dre04210:Apoptosis	4	0.07	4.00
	KEGG	↓ dre00190:Oxidative phosphorylation	6	0.1	2.40
	BP	↑ GO:0008380~RNA splicing	5	0.01	6.70
	BP	↑ GO:0006396~RNA processing	7	0.05	2.60
	BP	↑ GO:0006417~regulation of translation	3	0.05	7.90
	BP	↑ GO:0010608~posttranscriptional regulation of gene expression	3	0.08	6.30
	BP	↑ GO:0006397~mRNA processing	4	0.08	3.90
	BP	↑ GO:0009057~macromolecule catabolic process	6	0.08	2.50
	BP	↑ GO:0032268~regulation of cellular protein metabolic process	3	0.08	6.10
	BP	↑ GO:0016071~mRNA metabolic process	4	0.1	3.50
	KEGG	↑ dre04210:Apoptosis	4	0.03	5.80
	KEGG	↑ dre04340:Hedgehog signaling pathway	3	0.03	10.2
KEGG	↑ dre04510:Focal adhesion	6	0.04	3.10	
KEGG	↑ dre04520:Adherens junction	4	0.04	5.00	
30 days	BP	↓ GO:0060215~primitive hemopoiesis	2	0.05	41.9
	BP	↓ GO:0048514~blood vessel morphogenesis	3	0.07	6.50
	BP	↓ GO:0051082~unfolded protein binding	4	0.04	5.20
	KEGG	↓ dre04621:NOD-like receptor signaling pathway	3	0.05	8.30
	KEGG	↓ dre04144:Endocytosis	5	0.09	2.80
	BP	↑ GO:0006826~iron ion transport	3	0.00	35.7
	BP	↑ GO:0006099~tricarboxylic acid cycle	3	0.01	19.2
	BP	↑ GO:0046356~acetyl-CoA catabolic process	3	0.01	19.2
	BP	↑ GO:0009060~aerobic respiration	3	0.01	17.8
	BP	↑ GO:0006084~acetyl-CoA metabolic process	3	0.01	17.8
	BP	↑ GO:0006633~fatty acid biosynthetic process	3	0.02	14.7
	BP	↑ GO:0000041~transition metal ion transport	3	0.02	14.7
	BP	↑ GO:0055082~cellular chemical homeostasis	3	0.02	14.7
	BP	↑ GO:0006732~coenzyme metabolic process	4	0.02	6.80
	BP	↑ GO:0050801~ion homeostasis	3	0.02	11.9
	BP	↑ GO:0045333~cellular respiration	3	0.04	9.20
	BP	↑ GO:0051186~cofactor metabolic process	4	0.04	5.00
	BP	↑ GO:0055114~oxidation reduction	8	0.05	2.30
	BP	↑ GO:0006631~fatty acid metabolic process	3	0.05	8.30
	BP	↑ GO:0015980~energy derivation by oxidation of organic compounds	3	0.05	8.30
BP	↑ GO:0042246~tissue regeneration	2	0.08	23.8	
KEGG	↑ dre03320:PPAR signaling pathway	3	0.09	5.80	
KEGG	↑ dre00020:Citrate cycle (TCA cycle)	3	0.09	5.80	
60 days	KEGG	↓ dre04621:NOD-like receptor signaling pathway	3	0.02	12.0
	KEGG	↓ dre04210:Apoptosis	3	0.05	7.70
	KEGG	↓ dre04510:Focal adhesion	4	0.08	3.70
	BP	↑ GO:0009617~response to bacterium	3	0.02	13.2
	BP	↑ GO:0010604~positive regulation of macromolecule metabolic process	3	0.05	8.20
	BP	↑ GO:0009615~response to virus	2	0.07	26.3
	KEGG	↑ dre04210:Apoptosis	3	0.07	6.50
	KEGG	↑ dre04620:Toll-like receptor signaling pathway	3	0.07	6.50

LEGENDS OF FIGURES

Figure 1. Biomarker responses in mussels exposed to diclofenac (DIC), ibuprofen (IBU) and ketoprofen (KET) for 14, 30 and 60 days: lysosomal membrane stability, granulocytes/hyalinocytes ratio, phagocytosis capacity lipofuscin and neutral lipids content, Acyl CoA oxidase activity, DNA fragmentation and micronuclei frequency. Results are given as percentage variations compared to relative controls. Letters indicate significant differences among exposure times within the same treatment (One-Way ANOVA, Newman-Keuls post hoc comparisons).

Figure 2. Venn diagrams representing differentially expressed transcripts (total, up-regulated and down-regulated) in the comparisons CTRL vs KET at days 14, 30 and 60.

FIGURE 1

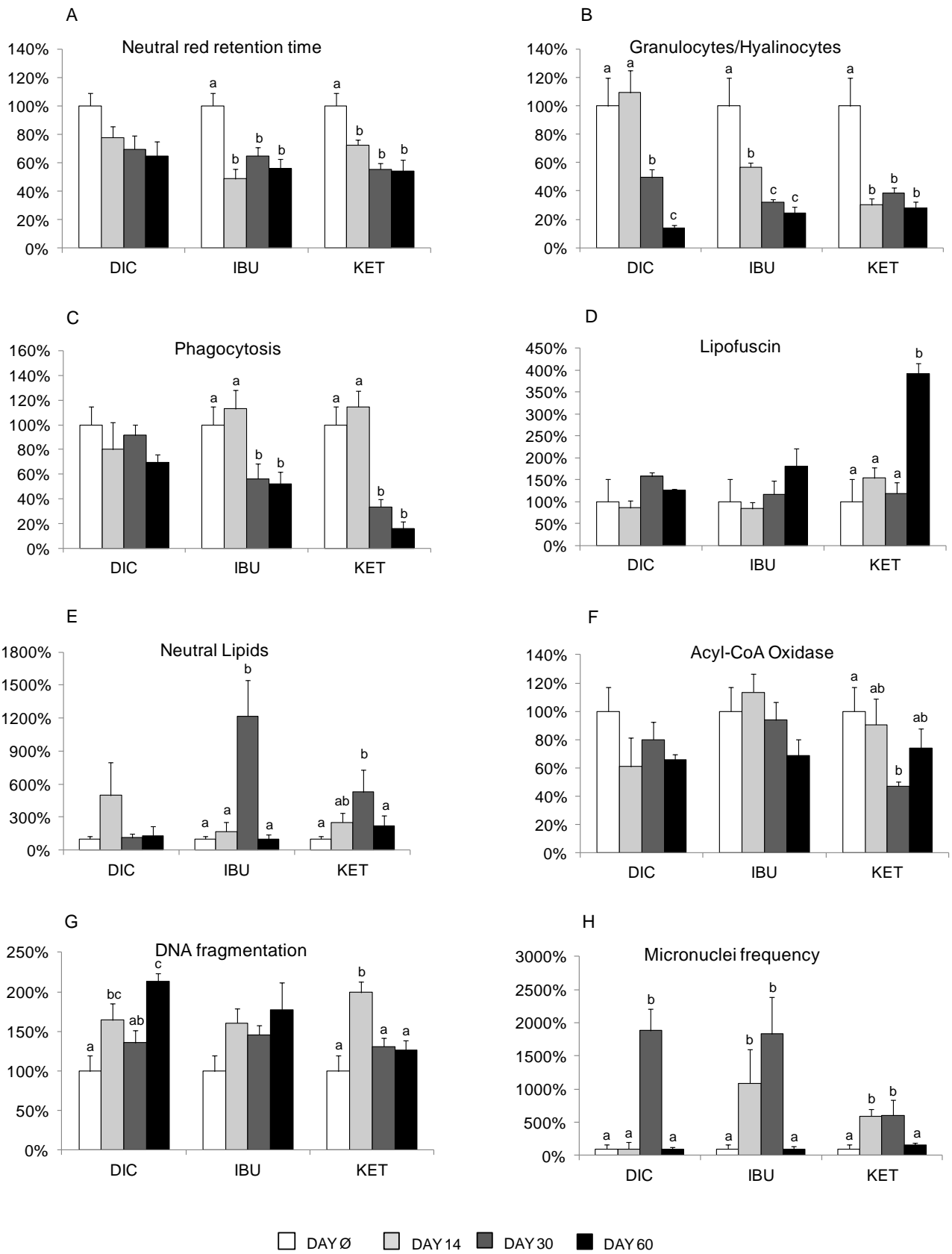
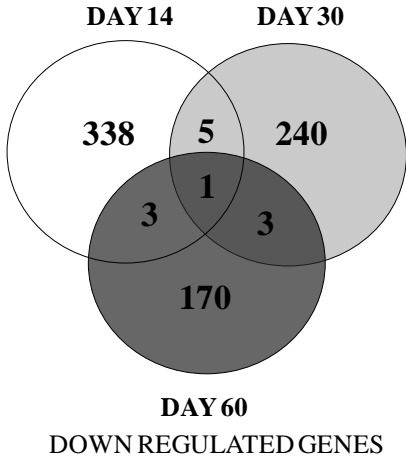
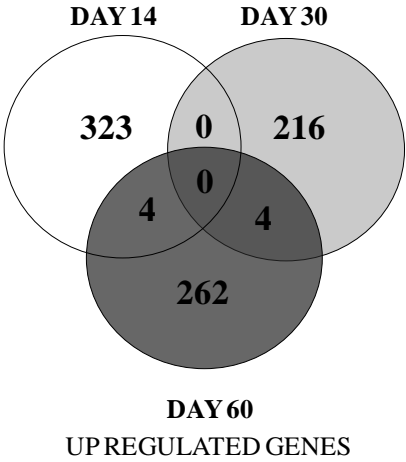
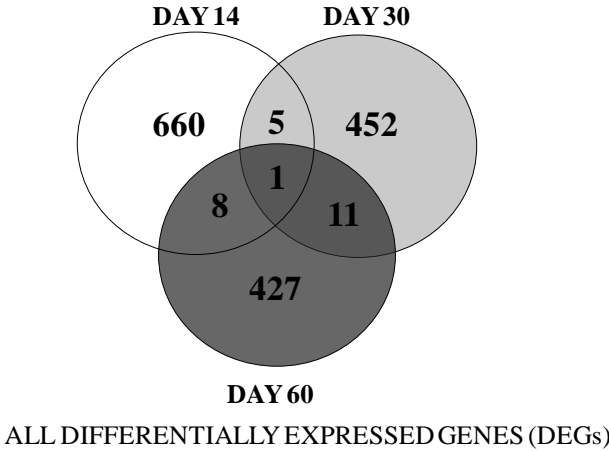


FIGURE 2



Supplementary Material

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