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

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Ancona November 24th, 2017

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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

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,

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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.

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 µg/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 (0.5-25µg/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 µg/L of KET and NIM, with significant changes of

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).

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

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

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

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

2. Materials and methods

2.1 Experimental design

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

Control organisms, *M. galloprovincialis* (5 ± 1 cm shell length), obtained from an unpolluted area of Central Adriatic Sea (Portonovo, Ancona), were maintained for 10 days at constant laboratory conditions (aerated seawater, 18 ± 1°C, salinity 37, pH 8.0 ± 0.5 and oxygen saturation >94%). Mussels were randomly sub-divided in twelve 20 L aquarium (60 mussels per tank, for a total of 720 specimens). The experimental design included nine tanks with organisms exposed to 2.5 µg/L of DIC, IBU and KET respectively (3 tanks for each treatment), and three solvent control tanks (CTRL) where methanol was added at the same concentration used in NSAIDs treatments (0.003%). The water in each tank was daily changed and concentrations of various drugs were re-established afterwards. Before water change, mussels were supplied with food (Zooplanktōs-s™, 50-300 µm), and no mortality was observed during the experiment. At days 14, 30 and 60, whole tissues were removed from 30 specimens for treatment (10 from each tank), pooled in 5 samples (each constituted by tissues of 6 organisms) and stored at −20°C until chemical analyses. Moreover,

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

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

2.2 Chemical analyses

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

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

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

2.3 Biological responses

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

2.4 Labelling, microarray hybridization and data acquisition

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

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

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

2.5 Statistical analyses

Analysis of variance was applied to test differences between treatments and exposure days (level of significance at $p < 0.001$). Cochram 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

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

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

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

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

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

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

3. Results

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

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

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

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

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

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

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

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

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

regulated autophagy modulator 2a DRAM2, *DNA fragmentation factor subunit beta DFFB*) and DNA damage (*DNA-damage inducible protein DDI1*, *nucleotide excision repair homolog MMS19*, *UV excision repair protein RAD23, growth arrest and DNA-damage-inducible alpha GADD45A*).

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

4. Discussion

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-pharmacoavigilance 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

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

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

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

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

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

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

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

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

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

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

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

5. Data accessibility

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

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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.

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 µg/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 (0.5-25µg/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 µg/L of KET and NIM, with significant changes of

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).

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

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

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

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

2. Materials and methods

2.1 Experimental design

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

Control organisms, *M. galloprovincialis* (5 ± 1 cm shell length), obtained from an unpolluted area of Central Adriatic Sea (Portonovo, Ancona), were maintained for 10 days at constant laboratory conditions (aerated seawater, 18 ± 1°C, salinity 37, pH 8.0 ± 0.5 and oxygen saturation >94%). Mussels were randomly sub-divided in twelve 20 L aquarium (60 mussels per tank, for a total of 720 specimens). The experimental design included nine tanks with organisms exposed to 2.5 µg/L of DIC, IBU and KET respectively (3 tanks for each treatment), and three solvent control tanks (CTRL) where methanol was added at the same concentration used in NSAIDs treatments (0.003%). The water in each tank was daily changed and concentrations of various drugs were re-established afterwards. Before water change, mussels were supplied with food (Zooplanktōs-s™, 50-300 µm), and no mortality was observed during the experiment. At days 14, 30 and 60, whole tissues were removed from 30 specimens for treatment (10 from each tank), pooled in 5 samples (each constituted by tissues of 6 organisms) and stored at −20°C until chemical analyses. Moreover,

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

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

2.2 Chemical analyses

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

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

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

2.3 Biological responses

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

2.4 Labelling, microarray hybridization and data acquisition

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

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

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

2.5 Statistical analyses

Analysis of variance was applied to test differences between treatments and exposure days (level of significance at $p < 0.001$). Cochram 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

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

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

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

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

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

3. Results

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

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

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

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

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

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

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

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

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

regulated autophagy modulator 2a DRAM2, *DNA fragmentation factor subunit beta DFFB*) and DNA damage (*DNA-damage inducible protein DDI1*, *nucleotide excision repair homolog MMS19*, *UV excision repair protein RAD23*, *growth arrest and DNA-damage-inducible alpha GADD45A*).

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

4. Discussion

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-pharmacoavigilance 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

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

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

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

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

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

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

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

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

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

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

5. Data accessibility

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

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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_2O_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.

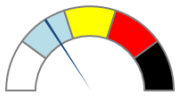
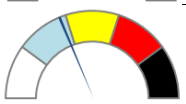
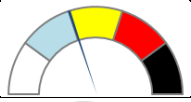
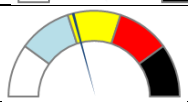
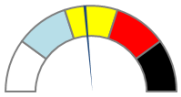
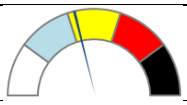
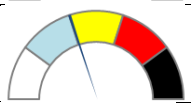
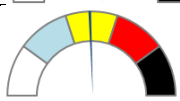
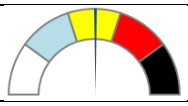
Compound	Day	Class of Hazard	Level
DIC	14	SLIGHT	
	30	SLIGHT	
	60	SLIGHT	
IBU	14	MODERATE	
	30	MODERATE	
	60	MODERATE	
KET	14	SLIGHT	
	30	MODERATE	
	60	MODERATE	

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

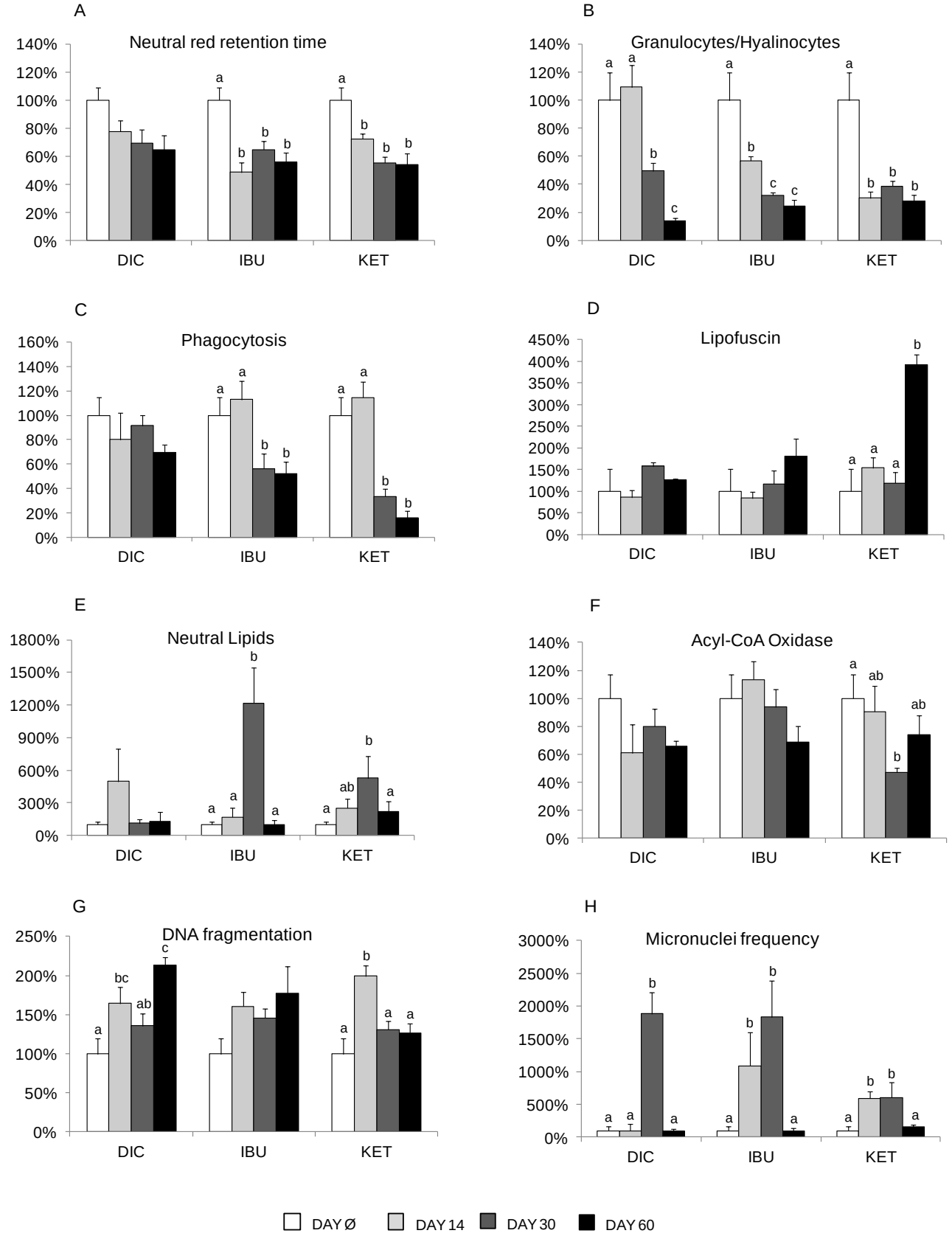
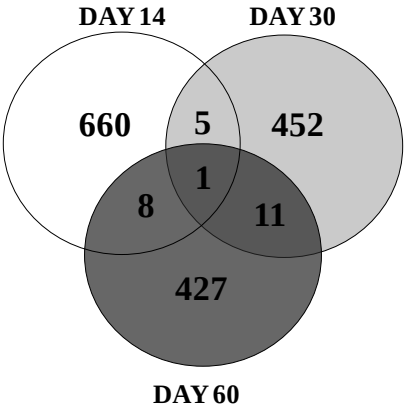
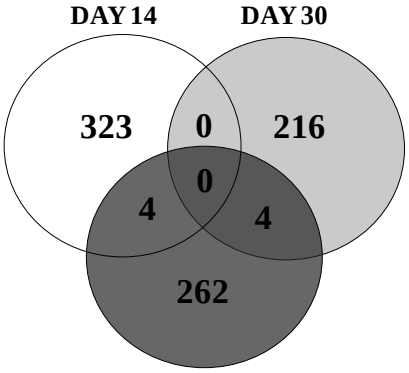

A
Neutral red retention time
B
Granulocytes/Hyalinocytes
C
Phagocytosis
D
Lipofuscin
E
Neutral Lipids
F
Acyl-CoA Oxidase
G
DNA fragmentation
H
Micronuclei frequency
DIC
IBU
KET
DAY Ø
DAY 14
DAY 30
DAY 60

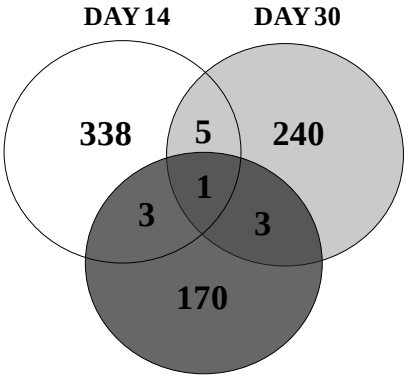
FIGURE 2



ALL DIFFERENTIALLY EXPRESSED GENES (DEGs)



UP REGULATED GENES



DOWN REGULATED GENES

Supplementary Material

Click here to download Supplementary Material: Supplementary Material SM1.docx

Supplementary Material 2

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