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ABSTRACT

The aim of the present investigation was to provide new insights on accumulation and possible adverse effects of various non-steroidal anti-inflammatory drugs (NSAIDs) in mussels, *Mytilus galloprovincialis*, exposed to an environmentally realistic concentration (0.5 µg/L) of individual compounds, Acetaminophen (AMP), Diclofenac (DIC), Ibuprofen (IBU), Ketoprofen (KET) or Nimesulide (NIM). The measurement of drugs in mussels tissues was integrated with both functional alterations at cellular level and transcriptomic responses. Results indicated the capability of mussels to accumulate DIC and NIM, while AMP, IBU and KET were always below detection limit. A large panel of ecotoxicological biomarkers revealed the early onset of alterations induced by tested NSAIDs on immunological responses, lipid metabolism and DNA integrity. The gene transcription analysis through DNA microarrays, supported cellular biomarker results, with clear modulation of a large number of genes involved in the arachidonic acid and lipid metabolism, immune responses, cell cycle and DNA repair. The overall results indicated an ecotoxicological concern for pharmaceuticals in *M. galloprovincialis*, with transcriptional responses appearing as sensitive exposure biomarkers at low levels of exposure: such changes, however, are not always paralleled by corresponding functional effects, suggesting caution when interpreting observed effects in terms of perturbed cellular pathways. Fascinating similarities can also be proposed in the mode of action of NSAIDs between bivalves and vertebrates species.

Keywords: Non-Steroidal Anti-Inflammatory Drugs, marine mussels, bioaccumulation, biomarkers, DNA-microarray

1. Introduction

Pharmaceutical compounds are increasingly documented as emerging contaminants in aquatic environments due to their massive use and the often limited removal by waste water treatment plants (Lolić et al., 2015; Petrie et al., 2014). Their ubiquitous presence in aquatic ecosystems and the evidence that these molecules are bioactive at low concentrations, raised the awareness of both scientific community and legislative authorities (Boxall et al., 2012; Santos et al., 2010). Effects of pharmaceuticals have been extensively characterized in humans and mammals, and it has been speculated whether they might affect non-target aquatic organisms with analogous mechanisms of action (MOA) on various species, tissues, cells, or biomolecules (Bebianno and Gonzalez-Rey, 2015; Boxall et al., 2012; Fent et al., 2006; Milan et al., 2013a). The major concern is related to the potential adverse effects on organisms permanently exposed to those compounds over their whole life cycle (Ankley et al., 2007; Fent et al., 2006).

Within the huge spectrum of environmental pharmaceuticals, non-steroidal anti-inflammatory drugs (NSAIDs) have been widely detected in aquatic ecosystems with concentrations ranging between few ng/L up to several µg/L in sewage treatment plants (STP) influents and effluents, rivers, groundwater, and coastal areas (Al Aukidy et al., 2012; Lolić et al., 2015; Pal et al., 2010). From initial studies on deleterious effects of pharmaceuticals in the environment assessed through acute toxicity tests (Santos et al., 2010), the ecotoxicological investigations have later focused on freshwater species, as those more directly exposed to compounds discharged by STP effluents into rivers (Gagné et al., 2005). For a long time, pharmaceuticals have not been considered to be harmful for oceans, due to the high dilution capacity of marine waters (Fabbri and Franzellitti, 2015). However, a recent study documented the occurrence of NSAIDs in wild populations of mussels, *Mytilus galloprovincialis*, collected in coastal areas of the Adriatic Sea (Mezzelani et al., 2016), converting this emerging issue into a potential threat also for marine ecosystems.

Investigations on pharmaceuticals in marine species are still limited, especially those addressing integrated questions on the environmental presence of these substances, their bioavailability to non-target species, and the onset of adverse effects at different levels of biological organization, i.e. from molecular alterations to organisms' health impairment. Such multidisciplinary approaches are fundamental within Environmental Risk Assessment (ERA) procedures, actually lacking for pharmaceuticals in the marine ecosystem. Mussels, *Mytilus spp.*, have been demonstrated to be suitable sentinel organisms for investigating accumulation and ecotoxicological effects of NSAIDs (Bebianno and Gonzalez-Rey, 2015; Mezzelani et al., 2016). Laboratory exposures to 25 µg/L of different compounds revealed the capability of these organisms

to accumulate Diclofenac (DIC), Ibuprofen (IBU) and Nimesulide (NIM), while Acetaminophen (AMP) and Ketoprofen (KET) were never detectable in mussels tissues (Mezzelani et al., 2016). On the other hand, all tested NSAIDs determined the clear onset of biochemical alterations, with modulation of lysosomal and immune system, a direct effect on stored energy reserves, and cellular alterations, potentially related to cell cycle modulation (Mezzelani et al., 2016).

Based on such results, the aim of the present study was to investigate whether anti-inflammatory pharmaceuticals exert their ecotoxicological potential even at much lower, environmentally realistic concentrations. In this respect, specimens of *M. galloprovincialis* were exposed for 14 days to individual drugs, respectively AMP, DIC, IBU, KET or NIM, each dosed at a concentration of 0.5 µg/L. This value is well within the range of those measured in different aquatic bodies, including marine environments and the Mediterranean Sea (Table 1). In some locations, detected levels were very low or even below detection limit, as it could be expected for any class of anthropogenic pollutants in pristine areas. However, concentrations spanning from hundreds up to thousands of ng/L were quite frequent and, worthy to note, the highest field concentrations measured in the Mediterranean Sea were greater that those used in the present study (200 µg/L for Acetaminophen, 1.5 µg/L for Diclofenac, 1.5 µg/L for Ibuprofen, 6 µg/L for Ketoprofen).

Accumulation of pharmaceuticals in tissues of exposed mussels was integrated with a large panel of cellular alterations such as immunological parameters, lysosomal membrane stability, antioxidant defences and oxidative stress biomarkers, the onset of genotoxicity, fatty acid metabolisms and neurotoxic effects. The overall results on biomarker responses were elaborated within a quantitative model (Sediqualsoft) which uses weighted criteria to summarize a cellular hazard index, based on the number, biological relevance and magnitude of the observed effects (Benedetti et al., 2012, 2014; Piva et al., 2011; Regoli et al., 2014). Measurements of functional effects at cellular level were further integrated with transcriptomic analyses in mussels exposed to KET and NIM, which had been previously shown to cause a similar ecotoxicological hazard at cellular level, despite their opposite behaviour in bioconcentration potential (Mezzelani et al., 2016). Although molecular changes are often considered to anticipate or support similar variations at corresponding biochemical or cellular pathways, several post-transcriptional and modulatory factors can influence links between different intracellular levels, which might be not necessarily present or easy to predict (Regoli et al., 2011; Giuliani et al., 2013; Regoli and Giuliani 2014).

Obtained results were expected to provide new insights on the putative MOA and ecotoxicological potential of NSAIDs in non-target marine organisms, thus representing an

additional step to support more comprehensive processes of environmental risk assessment and future management decisions on this new class of environmental pollutants.

2. Materials and methods

2.1 Experimental design

Chemicals, including Acetaminophen, Diclofenac, Ibuprofen, Ketoprofen and Nimesulide, were obtained from Sigma Aldrich. For each molecule a stock solution (10 mg/L in methanol) was prepared and stored at room temperature, while working solutions for mussels exposures were daily obtained by dilution in seawater.

Mussels, *M. galloprovincialis* (5 ± 1 cm shell length), were collected from a local farm (Ancona, Adriatic Sea) and acclimatized for 10 days to laboratory conditions with aerated seawater, at 18 ± 1°C, salinity 37, pH 8.0 ± 0.5 and oxygen saturation >94%. A total of 360 mussels were randomly distributed into six 20 L glass-tanks (60 mussels per tank); the experimental design included five tanks with organisms exposed to 0.5 µg/L of individual pharmaceuticals, AMP, DIC, IBU, KET or NIM respectively, and a solvent control tank (CTRL) where methanol was added at the same concentration used in the NSAIDs treatments (0.005%). Water was changed and all the molecules re-dosed on a daily basis: for this reason, we did not measure concentrations of NSAIDs in seawater, but we preferred to analyse the bioaccumulation of these pharmaceuticals in mussels tissues, considering this information much more relevant to interpret and discuss observed biological responses. No mortality of mussels was observed during the experiments.

After 14 days of exposure, 30 specimens for each treatment were dissected for chemical analyses, pooled in 5 samples (each containing whole tissues of 6 organisms) and stored at −20°C. Haemolymph, digestive glands and gills were rapidly removed from the remaining 30 specimens for each treatment, pooled in 10 samples (each constituted by tissues of 3 specimens) frozen in liquid nitrogen and maintained at -80°C for biochemical and histochemical analyses. An aliquot of haemolymph was also immediately processed for lysosomal membrane stability, phagocytosis activity and DNA damage; another aliquot was fixed in Carnoy's solution (3:1 methanol, acetic acid) for the microscopic evaluation of granulocytes-hyalinocytes ratio and micronuclei frequency. Four additional pools, each with digestive glands of three specimens, were prepared from CTRL, KET and NIM groups, for DNA microarray analysis.

2.2 Chemical analyses

Analytical methods and procedures for determination of NSAIDs in mussels tissues were carried out by high performance liquid chromatography (HPLC) according to previously validated

protocols (Mezzelani et al., 2016). Five replicates per group, each constituted by the whole tissues of 6 specimens were used to determine levels of accumulated NSAIDs. Acetic acid 0.1%, pH=3.26 was used as homogenization and extraction buffer (buffer 1) for DIC, IBU, KET and NIM, while ammonium phosphate 10 mM, pH=4.0 with citric acid 100 mM (buffer 2) for AMP. About 3 g of wet tissues were homogenized in 5 mL of buffers at room temperature for 20 minutes. After centrifugation at 4,500 xg for 30 minutes, samples were purified by Solid Phase Extraction (SPE); SPE tubes were pre-conditioned with 6 mL of methanol, followed by 18 mL of ultra-pure water and 12 mL of the buffers: samples were then loaded and, after washing with 12 mL of ultra-pure water, analytes were recovered using 3 mL of acetonitrile. Obtained samples were filtered, concentrated by SpeedVac and then centrifuged again at 12,000 xg for 20 minutes.

Chromatographic separations of DIC, IBU, KET and NIM were performed using an Agilent Infinity 1260 series system, with a C-18 Kinetex column (5 µm, 150 mm length, 4.6 mmID, Phenomenex, US). For KET and DIC a mobile phase composed by ultra-pure water (26%), acetonitrile (42%) and Buffer 1 (32%) was used under isocratic condition. The signals of KET and DIC were monitored and acquired with a diode array detector (DAD) at 250 nm and 276 nm respectively. Separations of IBU and NIM were performed on a gradient using ultra-pure water, acetonitrile and buffer 1 (from 35%:30%:35% to 0%:65%:35% for 23 minutes) on a C-18 Kinetex column (5 µm, 150 mm length, 4.6 mmID, Phenomenex, US). Detection of IBU was performed by fluorimetric detector (FD) with excitation/emission wavelengths at 230/294 nm, while NIM was detected using DAD monitoring at 298 nm. Separation of AMP was carried out by a C-18 Agilent Eclipse Plus column (3.5 µm, 100 mm length, 4.6 mmID) and a mobile phase composed by Buffer 2 (87.5%) and methanol (12.5%) under isocratic condition; signals were monitored and acquired using DAD at 248 nm. Concentrations of various NSAIDs were quantified by comparison with signals of pure standard solutions; due to the lack of appropriate Certified Standard Reference Materials (SRMs), the reproducibility, quality control and quality assurance were assessed analyzing a series of blank samples spiked with various concentrations of pure NSAIDs standards. An elevated recovery yield (CV<5%, n=10, ≥98%) was always ensured and checked; the minimum measurable amounts in mussels tissues were 1 ng/g dry weight (d.w.) for AMP, DIC and NIM, and 0.5 ng/g (d.w.) for IBU and KET.

2.3 Biological responses

Biomarkers in tissues of exposed mussels were measured through standardized protocols which included: microscopy analyses of haemocytes for lysosomal membrane stability through neutral red retention time (NRRT), granulocytes/hyalinocytes ratio (G/H), phagocytosis activity;

histochemical quantification of lipofuscin (LIPO) and neutral lipids (NL) on digestive gland cryostat sections; spectrophotometric determination of Acyl-CoA oxidase (ACOX), acetylcholinesterase (AChE) in haemolymph and gills, antioxidants defences (catalase, CAT, glutathione S-transferases, GST, Se-dependent and sum of Se-dependent and Se-independent glutathione peroxidases, GPx H_2O_2 and GPx CHP, glutathione reductase, GR, total glutathione, GSH) in digestive glands; gas-chromatographic assay of total antioxidant capacity (TOSC) toward peroxyl radicals (ROO·) and hydroxyl radicals (HO·) in digestive glands; electrophoretic and cytogenetic analysis of DNA integrity (DNA fragmentation and micronuclei, MN) in haemocytes. Detailed protocols are given in Supplementary Material 1 (SM1).

2.4 Labelling, microarray hybridization and data acquisition

Gene transcription analyses were performed using an 8x60K Agilent oligo-DNA microarray platform designed within the European project REPROSEED (REsearch project to improve PROduction of SEED of established and emerging bivalve species in European hatcheries). The DNA microarray platform has been recently described by Avio et al. (2015). Briefly, a total of 50,680 different *M. galloprovincialis* contigs have been represented by 59,997 probes. Information about sequencing, assembly, annotation and microarray design is summarized in Supplementary Material 2 (SM2). Probe sequences and other details on the microarray platform can be found in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GPL18667. Microarrays were synthesized in situ using the Agilent non-contact ink-jet technology including default positive and negative controls.

A total of 4 pools of digestive glands, each composed by tissues of 3 individuals, was analysed for gene expression analyses of CTRL, NIM and KET groups. Sample labelling and hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol with the Low Input Quick Amp Labeling kit. Full details about labelling, hybridization and data acquisition are reported in Supplementary Material 3 (SM3).

Raw gene expression data were deposited in the GEO database under accession number GSE66990. Normalization procedures included quantile normalization which always outperformed cyclic loess, and further adjustment by the parametric Combat in R to account for the between-experiments batch effects of the oligonucleotide microarray (Johnson et al., 2007). Normalized data were deposited in GEO archive under accession number GSE66990. The functional groups of analysed genes included: glycolysis/gluconeogenesis, citrate cycle, oxidative phosphorylation, glycerolipid metabolism, fatty acid metabolism, drug metabolism, regulation of transcription, spliceosome translation, Aminoacyl-tRNA biosynthesis, protein folding, proteolysis DNA repair,

lysosome, apoptosis, cell cycle, PPAR signaling pathway, immune response and arachidonic acid metabolism.

2.5 Statistical analyses

For chemical concentrations and biomarker responses, statistical analyses were performed using the Statistica Software (Ver 6.0, StatSoft, Tulsa, OK, USA). One way analysis of variance (ANOVA) was applied to test differences between various exposure groups; level of significance was set at $p < 0.05$, homogeneity of variance tested by Cochram C and mathematical transformation applied if necessary; post-hoc comparison (Newman-Keuls) was used to discriminate between means of values (n=5). Descriptive multivariate statistic (principal component analyses, PCA) was applied to biomarkers data for discriminating between different exposure conditions.

Data on biomarker responses were further elaborated within a previously developed quantitative and software-assisted model (Sediqualsoft), which summarizes large data-sets of heterogeneous data, providing a synthetic index of hazard: whole calculations, detailed flow-charts, rationale for weights, thresholds and expert judgements have been fully given elsewhere (Benedetti et al., 2014; Piva et al., 2011). Depending on species and tissue, the model assigns to each biomarker a "weight" based on the toxicological relevance of analyzed endpoint, and a "threshold" for changes of biological significance which consider both inductions and/or inhibitions of various responses. For every analysed biomarker, the variation measured in exposed organisms is compared to the threshold, then corrected for the weight of the response and the statistical significance of the difference compared to controls. Depending on the magnitude of the calculated effect, each biomarker response is assigned by the model to 1 of 5 classes of Effect (from Absent to Severe); the calculation of the Hazard Quotient for biomarkers (HQ_{BM}) does not consider the contribution of responses with an Effect lower or equal to threshold (Absent or Slight), calculates the average for those with an Effect up to two-fold compared to the threshold (Moderate), and adds the summation (Σ) for the responses more than 2 fold greater than the respective threshold, i.e. Major or Severe (Piva et al., 2011):

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

According to variations measured for various biomarkers, the model summarizes the level of cumulative HQ_{BM} in one of five classes of hazard for biomarkers (from Absent to Severe), maintaining scientifically sound information, while also being easy to read for non-expert stakeholders (Piva et al., 2011).

Statistical analyses of transcriptional results were performed on 45,887 out of 59,971 probes with signal higher than the background in 4 out of 12 analysed samples. The TIGR Multi Experiment Viewer 4.5.1 statistical software (TMeV; Saeed et al., 2003) was used to perform Principal Component Analysis (PCA) and T-test statistics (Bonferroni correction for multiple comparisons; p-value<0.01; 200 permutations) comparing CTRL to both KET and NIM groups. The resulting T-test genes lists were then filtered and 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). A more systematic functional interpretation of differentially transcribed genes was obtained through an enrichment analysis using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Huang et al., 2009). Since these databases contain functional annotation data for a limited number of species, transcripts of *M. galloprovincialis* were matched to *Danio rerio* Gene IDs using dedicated Blast searches performed with blastx (E-value < 10-5). The choice of *D. rerio* allowed the assignment of a putative homologue to a larger number of *M. galloprovincialis* transcripts (Avio et al., 2015), and was previously demonstrated an useful option for *Ruditapes philippinarum* functional analyses (Milan et al., 2011, 2013b). A functional annotation was obtained for genes differentially expressed in each T-test pairwise comparison, setting DAVID for gene count=2 and ease=0.1.

3. Results

Exposed mussels exhibited accumulation of DIC and NIM after 14 days, with tissue concentrations of 4.75 ± 5.30 ng/g d.w. and 8.00 ± 3.99 ng/g d.w., respectively (Table 2); levels of AMP, IBU and KET were always below the limit of detection (LOD).

Among immunological responses, lysosomal membrane stability was significantly reduced in organisms exposed to IBU (Fig.1A), while granulocytes-hyalinocytes ratio and phagocytosis activity increased in KET-exposed mussels (Fig. 1 B-C). Within biomarkers of cellular responses, a significant accumulation of lipofuscin in tertiary lysosomes was observed after exposures to DIC and NIM (Fig. 1D), and the neutral lipids content was lowered by all NSAIDs (Fig. 1E). No significant variations were measured for acyl-CoA oxidase and AChE in gills of exposed mussels (Fig. 1 F-G), but the latter activity was reduced in haemolymph after treatment with NIM (Fig. 1H).

Results on antioxidant defences and total oxyradical scavenging capacity are shown in Figure 2. Activities of catalase, glutathione S-transferases, glutathione peroxidases, glutathione reductase and levels of total glutathione showed only a few and not statistically significant variations between different exposure conditions (Fig. 2 A-F): such limited prooxidant effects were further supported by the lack of variations on the total oxyradical scavenging capacity toward both peroxyl (ROO•) and hydroxyl (•OH) radicals (Fig. 2 G-H). On the other hand, slight genotoxic effects were measured in terms of significant enhancement of DNA strand breaks for KET exposed organisms (Fig. 3A), and of micronuclei frequency after treatments with DIC, KET and NIM (Fig. 3B).

The principal component analysis (PCA) on the whole set of biomarkers produced a two dimensional pattern explaining 68.29% of the total variance. The plot score indicated a clear separation between control organisms (CTRL) and those exposed to DIC, KET and NIM (Fig.4). The parameters determining the separation along to the PC1 axis were those related to immunological alterations (granulocytes-hyalinocytes ratio, phagocytosis activity), antioxidant parameters (catalase, glutathione peroxidases, total oxyradical scavenging capacity toward peroxyl radicals) and genotoxic effects (micronuclei frequency and DNA fragmentation); accumulation of lipofuscin, acetylcholinesterase in haemolymph, glutathione S-transferases and total glutathione determined the separation of the groups along to the PC2 axis. The quantitative elaboration of these data based on the magnitude of variations observed for various biomarkers, their statistical significance and the toxicological relevance of each biological endpoint, summarized the hazard associated to biomarker responses as “Moderate” for organisms exposed to KET, “Slight” for AMP, DIC, IBU and NIM (Table 3).

Compared to such functional effects, pairwise comparisons of gene expression data revealed a total of 1,154 and 1,516 DEGs (T-test, $p<0,01$; 200 permutations; $FC>1.5$) in response to KET and NIM exposures, with 415 transcripts commonly up- or down-regulated in both comparisons (Fig. 5). Principal Component Analysis (PCA) was performed on DNA microarray data considering the entire set of probes (Fig. 6A), or the DEGs revealed in each comparison (Fig. 6B and 6C): all these elaborations revealed a clear separation between CTRL and exposed mussels along the x axis, while the clustering of NIM and KET groups would suggest similarities of gene expression profiles in response to such pharmaceuticals.

Functional interpretation of differentially transcribed genes was obtained through enrichment analysis with DAVID software (Table 4). Among the most interesting enriched Biological Processes (BP) and KEGG pathways, KET revealed “proteolysis” (represented by 25 DEGs), “monosaccharide metabolic process” (8 DEGs), “NOD-like receptor signaling pathway” (7

DEGs), “Apoptosis” (7 DEGs) and “p53 signaling pathway” (6 DEGs); enrichment analysis of DEGs in response to NIM revealed a total of 14 BP and 5 KEGG pathways including “response to stimulus” (43 DEGs), “oxidation reduction” (45 DEGs), “response to stress” (26 DEGs), “response to xenobiotic stimulus” (4 DEGs), “immune response” (6 DEGs), “Metabolism of xenobiotics by cytochrome P450” (5 DEGs), and “Apoptosis” (7 DEGs). Beside the above-mentioned BP and KEGG pathways, mussels exposed to both the NSAIDs showed the modulation of several genes involved in the arachidonic acid (AA) metabolism, carbohydrate and fatty acid metabolism, immune responses, DNA repair, apoptosis, cell cycle: full lists of enriched BP and KEGG pathways, and of significant genes involved in each process/pathways are reported in Supplementary Material 4 and 5 (SM4, SM5), respectively.

4. Discussion

Pharmaceuticals represent one of the most relevant groups of emerging contaminants in aquatic ecosystems (Zenker et al., 2014), and understanding their biological consequences on aquatic wildlife has become a research priority. Despite the marine environment has been considered as not affected by these pollutants for dilution, dispersion and degradation processes (Fabbri, 2015), recent studies demonstrated the presence of widely used non-steroidal anti-inflammatory pharmaceuticals in tissues of wild mussels populations from the Adriatic coast (Mezzelani et al., 2016). The onset of adverse effects in marine organisms exposed to different classes of neuroactive, anti-inflammatory and cardiovascular pharmaceuticals (Fabbri, 2015; Gonzalez-Rey and Bebianno, 2014; Mezzelani et al., 2016), further corroborated the need of a better characterization of this environmental risk.

The aim of the present study was to compare the sensitivity of biomarker responses previously highlighted in mussels exposed at 25 µg/L of various NSAIDs (Mezzelani et al., 2016), with a much lower, environmentally realistic exposure concentration (0.5 µg/L); higher values of these pharmaceuticals have already been reported for in the Mediterranean Sea (Togola and Budzinski, 2008; Loos et al., 2013; Nodler et al., 2014). The responsiveness of mussels was addressed in terms of bioaccumulation potential of these compounds, and integrating functional biological effects at cellular level with the earlier, transcriptional modulation of genes measured at molecular level. In our experiments, we considered nominal concentrations as exposure levels since water was changed and molecules re-dosed on a daily basis: however, particularly in field studies, measurements of these compounds in water may certainly contribute to better clarify the bioaccumulation processes in different non-target species.

Our results clearly highlighted the possibility for marine organisms to accumulate pharmaceuticals over a wide range of environmental levels. Tissue concentrations of DIC and NIM

increased after 14 days of exposure, suggesting a possible dose dependent behaviour, when compared to values measured in mussels exposed to 50 folds higher levels of the same pharmaceuticals (Mezzelani et al., 2016). On the other hand, IBU, previously accumulated after exposure to 25µg/L, remained below the detection limit in this study; both AMP and KET confirmed a lack of bioaccumulation in mussels tissues, independently on the exposure doses used either in the present or in the previous investigation (Mezzelani et al., 2016). However, an ecotoxicological concern was evidenced for all tested compounds even with limited or absent bioaccumulation, supporting the hypothesis that original compounds can be, at least partly, transformed by environmental or biological processes (Kümmerer, 2009; Bebianno and Gonzalez-Rey, 2015). The biological reactivity of NSAIDs without a specific marker of exposure may represent an important challenge for characterizing the environmental consequences of pharmaceuticals in field conditions.

Several biomarkers which appeared modulated after exposure to 25 µg/L (Mezzelani et al., 2016), were affected also in this study, although the magnitude of observed variations was lower and often not statistically significant. Destabilization of lysosomal membranes and enhanced accumulation of lipofuscin in tertiary lysosomes were caused by all NSAIDs at elevated concentrations (Mezzelani et al., 2016), while significant effects appeared in this study in mussels exposed to 0.5 µg/L of IBU, DIC or NIM. Similarly, the reduction of neutral lipids content and inhibition of ACOX observed at high doses of pharmaceuticals, were less marked at environmentally realistic levels. Overall, these results confirm the lysosomal compartment, lipid peroxidation and lipid metabolism as key cellular targets of tested drugs with an early onset of responsiveness at low exposure levels and increased effects at higher doses. The impairment of lysosomal membranes confirm a mode of action elicited by NSAIDs in aquatic invertebrates (Aguirre Martínez et al., 2013; Gonzalez-Rey and Bebianno, 2014; Parolini et al., 2011) while information on the potential role of those compounds in modulating lipid metabolisms, β-oxidation of the fatty acids and consumption of energy reserves, is still limited and partly supported by similar effects in rat liver and isolated mitochondria (Zhao et al., 1992). Organisms exposed to low levels of KET exhibited a significant increase of granulocytes-hyalinocytes ratio and of phagocytosis activity, which might reflect changes in cell cycle induced by NSAIDs, as previously demonstrated for mammals (Chang et al., 2009): despite the functionality of different haemocytes subpopulations is still debated for invertebrates, granulocytes seem mainly involved in phagocytosis, while hyalinocytes would be specialized in coagulation and encapsulation processes (Carballal et al., 1997; García- García et al., 2008; Giulianini et al., 2003; Gorbi et al., 2012, 2013).

Acetylcholinesterase activity was inhibited in haemolymph of mussels exposed to NIM while more limited effects were caused by other NSAIDs and in gills. The comparison of obtained results with those of previous investigations, suggests variable trends and biphasic variations of AChE in response to NSAIDs; a slight enzymatic enhancement in haemolymph was caused by 25 µg/L of AMP, DIC, KET and NIM, and by 250 ng/L of DIC in gills, while in the latter tissue AChE was inhibited by AMP (Gonzalez-Rey and Bebianno, 2014; Mezzelani et al., 2016). Considering the key role of acetylcholinesterase in terminating neurotransmission at cholinergic synapses and its modulation by a broad spectrum of xenobiotics, further studies are necessary to support the hypothesis that NSAIDs influence this enzyme through species- and tissue-specific mechanisms of action.

Responses of antioxidant defences toward low doses of NSAIDs were almost negligible compared to the slight but significant changes of catalase, glutathione S-transferases and TOSC toward hydroxyl radicals observed after exposure to 25 µg/L (Mezzelani et al., 2016). The lack of oxidative challenge observed in this study would confirm that prooxidant mechanisms do not represent the primary mode of action of these pharmaceuticals as already reported for marine mussels exposed to DIC and IBU (Gonzalez-Rey and Bebianno, 2014). Consistently with the onset of slight signals of cellular perturbations at environmental exposure doses, DNA fragmentation and frequency of micronuclei tended to increase in mussels exposed to KET, NIM and, to a lower extent, DIC. Although these effects were of limited magnitude, they still represent early warning signals for the same typology of alterations more clearly detected at higher doses of NSAIDs (Mezzelani et al., 2016; Parolini et al., 2011).

The multivariate PCA analysis of biomarkers provided a clear separation between control mussels and those exposed to various NSAIDs especially DIC, NIM and KET, confirming for the latter that the onset of biological effects is not necessarily related to a measurable accumulation of the compound. The biological relevance of observed effects was further highlighted by the elaboration of the overall results in a cellular hazard index. Although biomarkers can be useful in revealing early effects and mechanisms of action of environmental pollutants, their full potential is greatly enhanced when complex data obtained from multiple cellular pathways can be summarized in a cellular hazard or health index. In this respect, scientific literature provides several examples of integrative procedures based on the toxicological knowledge of biomarkers, which represent a valuable approach to present scientifically sound data in a user-friendly format, easy for non-expert stakeholders or decision-makers (Beliaeff and Burgeot, 2002; Piva et al., 2011; Broeg et al., 2005; Dagnino et al., 2008; Benedetti et al., 2012). Despite different algorithms and assumptions, such integrative procedures were shown to represent and added value, being all comparable in

discriminating altered health conditions (Marigómez et al., 2013). The calculations applied in the present investigation are part of a more complex Weight of Evidence model (Sediqualsoft) which uses weighted criteria to elaborate and integrate different typologies of data, as previously validated in several risk assessment studies (Benedetti et al., 2012, 2014; Regoli et al., 2014; Avio et al., 2015; Bebianno et al., 2015). When the biological significance of observed variations was quantified weighting the number, toxicological relevance and magnitude of effects, the cellular hazard was classified as "Slight" for AMP, DIC, IBU and NIM exposed organisms, "Moderate" for KET. The hazard index obtained after the exposure to 25μg/L was "Moderate" for all NSAIDs, further demonstrating that lower levels of pharmaceuticals affected the same cellular pathways at a quantitatively lower extent. Such elaboration of complex biological data in an hazard index allow easy comparisons between molecules, doses and exposure conditions, eliciting quantitatively different effects in non-target organisms and giving useful insights for a prioritization of pharmaceuticals within an Environmental Risk Assessment framework.

Considering the lack of accumulation for KET but the onset of cellular alterations in mussels exposed to this compound, it was of interest to further test the hypothesis of the possible lack of correlation between tissue concentrations of pharmaceuticals and biological effects at molecular level. Analyses on transcriptional profiles provided additional insights on activation of molecular mechanisms, suggesting a similar MOA by the comparison of gene expression profiles in mussels exposed to KET and NIM. In analogy with cellular biomarkers, pathways involved in immunological functions and fatty acid metabolism appeared among the primary responses to both the NSAIDs: a coordinated activation of innate immune defences was clearly indicated by the significant changes in a large number of genes which regulate lysosomal enzymes, toll-like receptors (TLRs), lectins and scavenger receptors (see SM 5).

Noteworthy, substantial effects were also observed in the regulation of genes involved in arachidonic acid (AA) metabolism. In mammals, NSAIDs act as inhibitors of cycloxygenase (COX), which is responsible for the formation of eicosanoids (prostaglandins, thromboxanes, leukotrienes) from AA. In the present study, the common up-regulation of PLA2 and down-regulation of TBXAS1, PGR1, PTGER4, and CYP4 (Table 4 and SM 5) suggest a COX-based mode of action for both KET and NIM, with an inhibition of eicosanoids synthesis in exposed mussels. A similar mechanism has already been suggested in other invertebrate species (Heckman et al., 2008; Milan et al. 2013a; Rowley et al., 2005) which corroborates the potential ecotoxicological effects of NSAIDs in marine organisms, considering the role of eicosanoids in reproduction (Stanley et al. 2000), gametogenesis (Heckman et al., 2008; Rowley et al., 2005) and larval settlement (Knight et al., 2000).

Molecular analyses revealed the down-regulation of *acyl-Coenzyme A oxidase 1* (ACOX1) and *acetyl-CoA acyltransferase 2* (ACAA2) in KET and NIM exposed mussels, respectively. These results confirm the earlier responsiveness of transcriptional modulation compared to cellular biomarkers at low exposure levels: reduced ACOX activities appeared as a trend in this study, but such, statistically significant functional effects were clearly observed in mussels at higher exposure doses (Mezzelani et al., 2016). The relationship between NSAIDs and lipid metabolism may be directly explained by the high affinity of these pharmaceuticals for peroxisome proliferator-activated receptors (PPARs) (Auwerx, 1999; Lehman et al., 1997).

The slight enhancement of DNA damage observed at cellular level in mussels exposed to KET and NIM, can be linked to the molecular up-regulation of RAD1 and SESN1. RAD1 is a component of cell cycle checkpoint complex that permits DNA repair preventing cell cycle progression (Volkmer and Karnitz, 1999). SESN1 is a stress-inducible gene with a role in the cellular response to DNA damage and oxidative stress, previously found to be up-regulated also in Manila clam exposed to ibuprofen (Milan et al., 2013a). Additional genes involved in control of the cell cycle and differentiation, such as *cyclin-dependent kinase 6* (CDK6), *cell division cycle 26 homolog* (CDC26), and *menage a trois homolog 1* (MNAT1), were significantly up-regulated in both KET and NIM exposed mussels, confirming the hypothesis of potential effects of NSAIDs on those pathways (Mezzelani et al., 2016).

Apoptosis was also found as an enriched KEGG in NIM and KET exposed mussels (SM4) with up-regulation of several genes, such as putative *baculoviral IAP repeat-containing 2* (BIRC2) and *X-linked inhibitor of apoptosis* (XIAP), *BCL2-associated athanogene 4* (BAG4), *B-cell CLL/lymphoma 2* (BCL2), and *BCL2-Like 1* (BCL2L1), and down-regulation of *caspase 2* (CASP2), *caspase 3* (CASP3) and *caspase 7* (CASP7). BIRC2 and XIAP are members of the apoptosis inhibitors family by interfering with the activation of caspase 3, 7 and 9, BCL2 encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death, while BAG4 belongs to the anti-apoptotic protein BAG1 family that may markedly increase the anti-cell death function of BCL2 (Takayama et al., 1995). Over-expression of BIRC2, XIAP, BCL2 and BAG4 was previously observed in clams exposed to ibuprofen (Milan et al 2013a), suggesting increased anti-apoptotic activity as a common response to NSAIDs in bivalves. Despite the still limited knowledge on molecular regulation of apoptosis in marine species, the observed transcriptional changes suggest similarities with the signaling pathways identified in other model organisms.

In conclusion, this study confirmed that some NSAIDs could be accumulated in mussels tissues also at very low, environmentally realistic levels. However, our results also demonstrated for the first time that ecotoxicological potential of pharmaceuticals is not necessarily reflected by tissue

concentrations of these compounds, suggesting the possibility of biotransformation and/or excretion mechanisms. Such a lack of correlation between accumulation and molecular-cellular effects is a well-known possibility in aquatic toxicology, like for polycyclic aromatic hydrocarbons in fish. The onset of biological effects was evident at molecular level, with clear modulation of a large number of genes involved in the arachidonic acid metabolism, immune responses, apoptosis, cell cycle and DNA repair. Molecular analyses supported the typology of functional variations observed among cellular biomarkers with immune system responses, lipid metabolism and genotoxic effects, as the main targets of NSAIDs in marine organisms. Similar data may represent the basis for the future development of an Adverse Outcome Pathway, designing appropriate experiments to provide clear-cut mechanistic representation of critical toxicological effects spanning over different levels of biological organization.

In the present work, alterations observed at various levels of biological organization exhibited a different quantitative modulation by dose of exposure. Transcriptional responses were more sensitive and activated even at low environmental levels of NSAIDs, when catalytic and cellular alterations were still of limited magnitude, becoming evident at higher exposure doses. In this respect, links between transcriptional and functional effects are not necessarily present, confirming the complexity of such relationships, which are modulated by several factors, including intensity and time of exposure.

Our data are obviously not conclusive, but the obtained results are promising and of potential interest to scientists, governments and industry. On one hand, they demonstrated biological responsiveness of pharmaceuticals in marine organisms, corroborating the utility of further studies, including exposures to multiple doses, mixtures and long-term periods, to clarify mode of action of NSAIDs in non-target species, and to explore possible links between cellular effects, physiological and ecological consequences. On the other hand, the present study also highlights the urgency to include pharmaceutical contaminants in normative guidelines and monitoring protocols for the assessment of good ecological status of marine environment, and the recommendation to consider ecotoxicological effects when designing new active molecules or prescribing already existing commercial compounds.

5. Data accessibility

The following link has been created to allow review of record GSE66990 while it remains in private status:

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=qnshskicbbehdmb&acc=GSE66990

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7. References

Aguirre-Martínez, G.V., Buratti, S., Fabbri, E., Del Valls, A.T., Martín-Díaz, M.L., 2013. Using lysosomal membrane stability of haemocytes in *Ruditapes philippinarum* as a biomarker of cellular stress to assess contamination by caffeine, ibuprofen, carbamazepine and novobiocin. J. Environ. Sci. 25, 1408-1418.

Al Aukidy, M., Verlicchi, P., Jelic, A., Petrovic, M., Barcelò, D., 2012. Monitoring release of pharmaceutical compounds: occurrence and environmental risk assessment of two WWTP effluents and their receiving bodies in the Po Valley, Italy. Sci. Total Environ. 438, 15-25.

Andreozzi, R., Marotta, R., Nicklas, P., 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere 50, 1319-1330.

Ankley, G.T., Brooks, B.W., Huggett, D.B., Sumpter, J.P., 2007. Repeating history: Pharmaceuticals in the environment. Environ. Sci. Technol. 41, 8211-8217.

Ashton, D., Hilton, M., Thomas, K.V., 2004. Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. Sci. Total Environ. 333, 167-184.

Auwerx, J., 1999. PPAR gamma, the ultimate thrifty gene. Diabetologia. 42(9), 1033-1049.

Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., Pauletto, M., Bargelloni, L., Regoli, F., 2015. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. Environ. Pollut. 198, 211-222.

Bayen, S., Zhang, H., Desai, M.M., Ooi, S.K., Kelly, B.C., 2013. Occurrence and distribution of pharmaceutically active and endocrine disrupting compounds in Singapore's marine environment: influence of hydrodynamics and physical–chemical properties. Environ. Pollut. 182, 1-8.

Bebianno, M.J., Gonzalez-Rey M., 2015. Ecotoxicological Risk of Personal Care Products and Pharmaceuticals in Claude Amiard-Triquet, Jean-Claude Amiard and Catherine Mouneyrac, Aquatic Ecotoxicology, Advancing Tools for Dealing with Emerging Risks. Academic Press Ch. 16, 383-416.

Beliaeff, B., Burgeot, T., 2002. Integrated biomarker response: a useful tool for ecological risk assessment. Environ. Toxicol. Chem. 21, 1316-1322.

Bendz, D., Paxéus, N.A., Ginn, T.R., Loge, F.J., 2005. Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Höje River in Sweden. J. Hazard. Mater. 122, 195-204.

Benedetti, M., Ciaprini, F., Piva, F., Onorati, F., Fattorini, D., Notti, A., Ausili, A., Regoli, F., 2012. A multidisciplinary weight of evidence approach toward polluted sediments: integrating sediment chemistry, bioavailability, biomarkers responses and bioassays. Environ. Int. 38, 17-28.

Benedetti, M., Gorbi, S., Fattorini, D., D'Errico, G., Piva, F., Pacitti, D., Regoli F., 2014. Environmental hazards from natural hydrocarbons seepage: integrated classification risk from sediment chemistry, bioavailability and biomarker responses in sentinel species Environ. Pollut. 185, 116-126.

Boxall, A.B., Rudd, M.A., Brooks, B.W., Caldwell, D.J., *et al*. 2012. Pharmaceuticals and personal care products in the environment: what are the big questions? Environ. Health Perspect. 120, 1221-1229.

Broeg, K., Westernhagen, H.V., Zander, S., Körting, W., Koehler, A., 2005. The ‘‘bioeffect assessment index’’ – a concept for the quantification of effects of marine pollution by an integrated biomarker approach. Mar. Pollut. Bull. 50, 495-503.

Buser, H.R., Poiger, T., Müller, M.D., 1999. Occurrence and environmental behavior of the pharmaceutical drug ibuprofen in surface waters and in wastewater. Environ. Sci. Technol. 33, 2529-2535.

Carballal, M.J., Lopez, M.C., Azevedo, C., Villalba, A., 1997. Hemolymph cell types of the mussel *Mytilus galloprovincialis*. Dis. Aquat. Org. 29, 127-135.

Carmona, E., Andreu, V., Picó, Y., 2014. Occurrence of acidic pharmaceuticals and personal care products in Turia River Basin: from waste to drinking water. Sci. Total Environ. 484, 53-63.

Castiglioni, S., Bagnati, R., Calamari, D., Fanelli, R., Zuccato, E., 2005. A multi residue analytical method using solid-phase extraction and high-pressure chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters. J. Chromatogr. A 1092, 206-215.

Chang, J.K., Li, C.J., Liao, H.J., Wang, C.K., Wang, G.J., Ho, M.L., 2009. Anti-inflammatory drugs suppress proliferation and induce apoptosis through altering expressions of cell cycle regulators and pro-apoptotic factors in cultured human osteoblasts. Toxicology. 258, (2-3) 148-156.

Dagnino, A., Sforzini, S., Dondero, F., Fenoglio, S., Bona, E., Jensen, J., Viarengo, A., 2008. A ‘‘Weight-of-Evidence’’ approach for the integration of environmental ‘‘Triad’’ data to assess ecological risk and biological vulnerability. Integr. Environ. Assess. Manage. 4, 314-326.

Fabbri, E., 2015. Pharmaceuticals in the environment: expected and unexpected effects on aquatic fauna. Ann. N. Y. Acad. Sci. 1340, 20-28.

Fabbri, E., Franzellitti, S., 2015. Human pharmaceuticals in the marine environment: focus on exposure and biological effects in animal species. Environ. Toxicol. Chem. 35 (4), 799-812.

Fang, T.H., Nan, F.H, Chin, T.S, Feng, H.M. 2012. The occurrence and distribution of pharmaceutical compounds in the effluents of a major sewage treatment plant in Northern Taiwan and the receiving coastal waters. Mar. Pollut. Bull. 64, 1435-1444.

Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122-159.

Ferrari, F., Gallipoli, A., Balderacchi, M., Ulaszewska, M.M., Capri, E., Trevisan, M., 2011. Exposure of the Main Italian River Basin to Pharmaceuticals. J. Toxicol 2011, 989270.

Gagné, F., Berube, E., Fournier, M., Blaise, C., 2005. Inflammatory properties of municipal effluents to *Elliptio complanata* mussels: lack of effects from anti-inflammatory drugs. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 141, 332-337.

García-García, E., Prado-Álvarez, M., Novoa, B., Figueras, A., Rosales, C., 2008. Immune responses of mussel hemocyte subpopulations are differentially regulated by enzymes of the PI 3-K, PKC, and ERK kinase families. Dev. Comp. Immunol. 32, 637-653.

Giuliani, M.E., Benedetti, M., Arukwe, A., Regoli, F., 2013. Transcriptional and catalytic responses of antioxidant and biotransformation pathways in mussels, *Mytilus galloprovincialis*, exposed to chemical mixtures. Aquat. Toxicol. 134–135, 120–127.

Giulianini, P.G., Bertolo, F., Battistella, S., Amirante, G.A., 2003. Ultrastructure of the hemocytes of *Cetonischema aeruginosa* larvae (Coleoptera, Scarabaeidae): involvement of both granulocytes and oenocytoids in vivo phagocytosis. Tissue Cell 35, 243-251.

Gómez, M.J., Martínez Bueno, M.J., Lacorte, S., Fernández-Alba, A.R., Agüera A., 2007. Pilot survey monitoring pharmaceuticals and related compounds in a sewage treatment plant located on the Mediterranean coast. Chemosphere 66, 993-1002.

Gonzalez-Rey, M., Bebianno, M.J., 2014. Effects of non-steroidal anti-inflammatory drug (NSAID) diclofenac exposure in mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 148, 221-230.

Gonzalez-Rey, M., Tapie, N., Le Menach, K., Dévier, M.H., Budzinski, H., Bebianno, M.J., 2015. Occurrence of APIS and pesticides in aquatic systems. Mar. Pollut. Bull. 96, 384-400.

Gorbi, S., Bocchetti, R., Binelli, A., Bacchiocchi, S., Orletti, R., Nanetti, L., Raffaelli, F., Vignini, A., Accoroni, S., Totti, C., Regoli, F., 2012. Biological effects of palytoxinlike compounds from *Ostreopsis cf. ovata*: a multibiomarkers approach with mussels *Mytilus galloprovincialis*. Chemosphere 89, 623-632.

Gorbi, S., Avio, C.G., Benedetti, M., Totti, C., Accoroni, S., Pichierri, S., Bacchiocchi, S., Orletti, R., Graziosi, T., Regoli, F., 2013. Effects of harmful dinoflagellate *Ostreopsis cf. ovata* exposure on immunological, histological and oxidative responses of mussels *Mytilus galloprovincialis*. Fish Shellfish Immunol. 35, 941-950.

Gros, M., Petrovic, M., Barceló, D., 2006. Development of a multi-residue analytical methodology based on liquid chromatography-tandem mass spectrometry (LC–MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters. Talanta 70, 678-690.

Gros, M., Rodríguez-Mozaz, S., Barceló, D., 2012. Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. J. Chromatogr. A 1248, 104-121.

Heckmann, L.H., Sibly, R.M., Connon, R., Hooper, H.L., Hutchinson, T.H., Maund, S.J., Hill, C.J., Bouetard, A., Callaghan, A., 2008. Systems biology meets stress ecology: linking molecular and organismal stress responses in *Daphnia magna*. Genome Biol. 9, R40.

Heberer, T., Dünnbier, U., Reilich, C., Stan, H.J., 1997. Detection of drugs and drug metabolites in ground water samples of a drinking water treatment plant. Fresen. Environ. Bull. 6, 438-443.

Heberer, T., Reddersen, K., 2001. Occurrence and fate of pharmaceutical residues in the aquatic system of Berlin as an example for urban ecosystems. Proceedings of the Second International Conference on Pharmaceuticals and Endocrine Disrupting Chemicals in Water. Minneapolis, USA.

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44-57.

Johnson, W.E., Li, C., Rabinovic, A., 2007. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 8, 118-127.

Kim, Y., Choi, K., Jung, J., Park, S., Kim, P.G., Park, J., 2007. Aquatic toxicity of acetaminophen, carbamazepine, cimetidine, diltiazem and six major sulfonamides, and their potential ecological risks in Korea. Environ. Int. 33, 370-375.

Knight, J., Rowley, A.F., Yamazaki, M., Clare, A.S., 2000. Eicosanoids are modulators of larval settlement in the barnacle, *Balanus amphitrite*. J. Mar. Biol. Assn UK 80, 113-117.

Kümmerer, K., 2009. The presence of pharmaceuticals in the environment due to human use - present knowledge and future challenges. J. Environ. Manag. 90, 2354-2366.

Lacey, C., Basha, S., Morrisey, A., Tobin, J.M., 2012. Occurrence of pharmaceutical compounds in wastewater process streams in Dublin, Ireland. Environ. Monit. Asses. 184, 1049-1062.

Lee, H.B., Peart, T.E., Svoboda M.L., 2005. Determination of endocrine-disrupting phenols, acidic pharmaceuticals, and personal-care products in sewage by solid-phase extraction and gas chromatography-mass spectrometry. J. Chromatogr. A 1094, 122-129.

Lehmann, J.M., Lenhard, J.M., Oliver, B.B., Ringold, G.M., Kliewer, S.A., 1997. Peroxisome proliferator-activated receptors a and g are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J. Biol. Chem. 272, 3406-3410.

Lin, A.Y.C., Tsai, Y.T., 2009. Occurrence of pharmaceuticals in Taiwan's surface waters: Impact of waste streams from hospitals and pharmaceutical production facilities. Sci. Total Environ. 407, 3793-3802.

Lolić, A., Paíga, P., Santos, L.H.M.L.M., Ramos, S., Correia, M., Delerue-Matos, C., 2015. Assessment of non-steroidal anti-inflammatory and analgesic pharmaceuticals in seawaters of North of Portugal: occurrence and environmental risk. Sci. Total Environ. 508, 240-250.

Loos, R., Carvalho, R., Antonio, D.C., Cornero, S., Locoro, G., Tavazzi, S., Paracchini, B., Ghiani, M., Lettieri, T., Blaha, L., Jarosova, B., Voorspoels, S., Servaes, K., Haglund, P., Fick, J., Lindberg, R.H., Schwesig, D., Gawlik, B.M., 2013. EU wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. Water Res. 47, 6475-6487.

Loos, R., Tavazzi, S., Paracchini, B., Canuti, E., Weissteiner, C., 2013. Analysis of polar organic contaminants in surface water of the northern Adriatic Sea by solid-phase extraction followed by ultrahigh-pressure liquid chromatography-QTRAP(A (R)) MS using a hybrid triple-quadrupole linear ion trap instrument. Anal. Bioanal. Chem. 405, 5875-5885.

Marigómez, I., Zorita, I., Izagirre, U., Ortiz-Zarragoitia, M., Navarro, P., Etxebarria, N., Orbea, A., Soto, M., Cajaraville, M.P., 2013. Combined use of native and caged mussels to assess biological effects of pollution through the integrative biomarker approach. Aquat. Toxicol. 136-137, 32-48.

McEneff, G., Barron, L., Kelleher, B., Paull, B., Quinn, B., 2014. A year-long study of the spatial occurrence and relative distribution of pharmaceutical residues in sewage effluent, receiving marine waters and marine bivalves. Sci. Total. Environ. 476-477, 317-326.

Metcalfe, C.D., Koenig, B.G., Bennie, D.T., Servos, M., Ternes, T.A., Hirsch, R., 2003. Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. Environ. Toxicol. Chem. 22, 2872-2880.

Mezzelani, M., Gorbi, S., Da Ros, Z., Fattorini, D., d'Errico, G., Milan, M., Bargelloni, L., Regoli F., 2016. Ecotoxicological potential of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in marine organisms: bioavailability, biomarkers and natural occurrence in *Mytilus galloprovincialis*. Mar. Environ. Res. In press. http:// dx.doi.org/10.1016/j.marenvres.2016.03.005

Milan, M., Coppe, A., Reinhardt, R., Cancela, L.M., Leite, R.B., Saavedra, C., Ciofi, C., Chelazzi, G., Patarnello, T., Bortoluzzi, S., Bargelloni, L., 2011. Trascriptome sequencing and microarray development for the Manila clam, *Ruditapes philippinarum*: genomic tools for environmental monitoring. BMC Gen. 12, 234.

Milan, M., Pauletto, M., Patarnello, T., Bargelloni, L., Marin, M.G., Matozzo, V., 2013a. Gene transcription and biomarker responses in the clam *Ruditapes philippinarum* after exposure to ibuprofen. Aquat. Toxicol. 126, 17-29.

Milan, M., Ferraresso, S., Ciofi, C., Chelazzi, G., Carrer, C., Ferrari, G., Pavan, L., Patarnello, T., Bargelloni, L., 2013b. Exploring the effects of seasonality and chemical pollution on the hepatopancreas transcriptome of the Manila clam. Mol. Ecol. 22 (8), 2157-2172.

Nakada, N., Tanishima, T., Shinohara, H., Kiri, K., Takada, H., 2006. Pharmaceutical chemicals and endocrine disrupters in municipal wastewater in Tokyo and their removal during activated sludge treatment. Water Res. 40, 3297–3303.

Nebot, C., Gibb, S.W., Boyd, K.G., 2007. Quantification of human pharmaceuticals in water samples by high performance liquid chromatography–tandem mass spectrometry. Anal. Chim. Acta. 598, 87-94.

Nödler, K., Voutsa, D., Licha, T., 2014. Polar organic micropollutants in the coastal environment of different marine systems. Mar. Pollut. Bull. 85, 50-59.

Paíga, P., Santos, H.M.L.M., Amorim, C.G., Araújo, A.N., Montenegro, M.C., Pena, A., Delerue-Matos, C., 2013. Pilot monitoring study of ibuprofen in surface waters of north of Portugal. Environ. Sci. Pollut. Res. 20, 2410-2420.

Pailler, J.Y., Krein, A., Pfister, L., Hoffmann, L., Guignard, C., 2009. Solid phase extraction coupled to liquid chromatography-tandem mass spectrometry analysis of sulfonamides, tetracyclines, analgesics and hormones in surface water and wastewater in Luxembourg. Sci. Total Environ. 407, 4736-4743.

Pal, A., Gin, A.Y.C., Reinhard, M., 2010. Impacts of emerging organic contaminants on freshwater resources: review of recent occurrences, sources, fate and effects. Sci. Total Environ. 8, 6062-6069.

Parolini, M., Binelli, A., Provini, A., 2011. Chronic effects induced by ibuprofen on the freshwater bivalve *Dreissena polymorpha*. Ecotoxicol. Environ. Saf. 74, 1586-1594.

Petrie, B., Barden, R., Kasprzyk-Hordern, B., 2014. A review on emerging contaminants in wastewaters and the environment: current knowledge, understudied areas and recommendations for future monitoring. Water Res. 30, 1-25.

Piva, F., Ciaprini, F., Onorati, F., Benedetti, M., Fattorini, D., Ausili, A., Regoli, F, 2011. Assessing sediment hazard through a Weight Of Evidence approach with bioindicator organisms: a practical model to elaborate data from sediment chemistry, bioavailability, biomarkers and ecotoxicological bioassays. Chemosphere 83, 475-485.

Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar. Environ. Res 93, 106-117.

Regoli, F., Giuliani, M.E., Benedetti, M., Arukwe, A., 2011. Molecular and biochemical biomarkers in environmental monitoring: A comparison of biotransformation and antioxidant defence systems in multiple tissues. Aquat. Toxicol. 105S (2011), 56– 66.

Regoli, F., Pellegrini, D., Cicero, A.M., Nigro, M., Benedetti, M., Gorbi, S., Fattorini, D., D'Errico, G., Di Carlo, M., Nardi, A., Gaion, A., Scuderi, A., Giuliani, S., Romanelli, G., Berto, D., Trabucco, B., Bernardeschi, M., Guidi, P., Scarcella, V., Frenzilli G., 2014. A multidisciplinary weight of evidence approach for environmental risk assessment at the Costa Concordia wreck: integrative indices from Mussel Watch. Mar. Environ. Res. 96, 92-104.

Roberts, P.H., Bersuder, P., 2006. Analysis of OSPAR priority pharmaceuticals using high - performance liquid chromatography-electrospray ionization tandem mass spectrometry. J. Chromatogr. A 1134, 143–150.

Rodriguez-Navas, C., Bjoerklund, E., Bak, S.A., Hansen, M., Krogh, K.A., Maya, F., Forteza, R., Cerda, V., 2013. Pollution pathways of pharmaceutical residues in the aquatic environment on the Island of Mallorca, Spain. Arch. Environ. Contam. Toxicol. 65,56-66.

Rowley, A.E., Vogan, C.L., Taylor, G.W., Clare, A.S., 2005. Prostaglandins in non-insectan invertebrates: recent insights and unsolved problems. J. Exp. Biol. 208 (Pt 1), 3-14.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., Quackenbush, J., 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34, 374-378.

Salgado, R., Noronha, J. P., Oehmen, A., Carvalho, G., Reis, M. A. M., 2010. Analysis of 65 pharmaceuticals and personal care products in 5 wastewater treatment plants in Portugal using a simplified analytical methodology. Water Sci. Technol. 62(12), 2862-2871.

Santos, J.L., Aparicio, I., Alonso, E., Callejón, M., 2005. Simultaneous determination of pharmaceutically active compounds in wastewater samples by solid phase extraction and high-performance liquid chromatography with diode array and fluorescence detectors. Anal. Chim. Acta. 550, 116-122.

Santos, L.H.M.L.M., Araújo, A.N., Fachini, A., Pena, A., Delerue-Matos, C., Montenegro, M.C.B.S.M., 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. J. Hazard Mater. 175,45-95.

Stanley, D.W., 2000. Eicosanoids in Invertebrate Signal Transduction Systems. Princeton University Press, Princeton, NJ.

Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J.A., Reed, J.C. 1995. Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. Cell 80(2), 279-284.

Tauxe-Wuersch, A., De Alencastro, L.F., Grandjean, D., Tarradellas, J., 2005. Occurrence of several acidic drugs in sewage treatment plants in Switzerland and risk assessment. Water Res. 39, 1761-1772.

Ternes T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245-3260.

Thomas, K.V., Hilton, M.J., 2004. The occurrence of selected human pharmaceutical compounds in UK estuaries. Mar. Pollut. Bull. 49, 436-444.

Thomas, P.M., Foster, G.D., 2004. Determination of non steroidal anti-inflammatory drugs, caffeine, and triclosan in wastewater by gas chromatography-mass spectrometry, J. Environ. Sci. Health. Part A Toxic/Hazard. Subst. Environ. Eng. A 39, 1969–1978.

Togola, A., Budzinski, H., 2008. Multi-residue analysis of pharmaceutical compounds in aqueous samples. J. Chromatogr. A 1177, 150-158.

Volkmer, E., Karnitz, L.M., 1999. Human homologs of *Schizosaccharomyces pombe* rad1, hus1, and rad9 form a DNA damage-responsive protein complex. J. Biol. Chem. 274,567-570.

Wang, L., Ying, G.G., Zhao, J.L., Yang, X.B., Chen, F., Tao, R., Liu, S., Zhou, L.J., 2010. Occurrence and risk assessment of acidic pharmaceuticals in the Yellow River, Hai River and Liao River of north China. Sci. Total Environ. 408, 3139-3147.

Weigel, S., Berger, U., Jensen, E., Kallenborn, R., Thoresen, H., Hühnerfuss, H., 2004. Determination of selected pharmaceuticals and caffeine in sewage and seawater from Tromsø/ Norway with emphasis on ibuprofen and its metabolites. Chemosphere 56, 583-92.

Wu, J., Qian, X., Yang, Z., Zhang, L., 2010. Study on the matrix effect in the determination of selected pharmaceutical residues in seawater by solid-phase extraction and ultra-high performance liquid chromatography-electrospray ionization low-energy collision induced dissociation tandem mass spectrometry. J. Chromatogr. A 1217, 1471-1475.

Zenker, A., Cicero, M.R., Prestinaci, F., Bottoni, P., Carere, M., 2014. Bioaccumulation and biomagnification potential of pharmaceuticals with a focus to the aquatic environment. J. Environ. Manage. 133, 378-387.

Zhao, B., Geisslinger, G., Hall, I., Day, R.O., Williams, K.M., 1992. The effect of enantiomers of ibuprofen and flurbiprofen on the b-oxidation of palmitate in the rat, Chirality 4, 137-141.

Zorita, S., Mårtensson, L., Mathiasson, L., 2009. Occurrence and removal of pharmaceuticalsin a municipal sewage treatment system in the south of Sweden. Sci. Total Environ. 407, 2760-2770.

Zuccato, E., Castiglioni, S., Fanelli, R., Reitano, G., Bagnati, R., Chiabrando, C., Pomati, F., Rossetti, C., Calamari, D., 2006. Pharmaceuticals in the environment in Italy: causes, occurrence, effects and control, Environ. Sci. Pollut. Res. 13, 15-21.

TABLES

Table 1. Environmental concentrations of Acetaminophen, Diclofenac, Ibuprofen, Ketoprofen and Nimesulide measured in different aquatic bodies.

Compound	Country	Environment	Concentration ng/L	References
Acetaminophen	France	Mediterranean Sea	200000	Togola and Budzinki, 2008
	Greece	Aegean See	2983	Nödler et al., 2014
	Portugal	Arade Estuary	88	Gonzalez Rey et al., 2015
	Spain	Hospital Effluent	500	Kim et al., 2007
	Spain	STP Effluent	32-4200	Kim et al., 2007
	Spain	Mediterranean Sea	23	Gros et al., 2012
	Taiwan	Hospital Effluent	62	Kim et al., 2007
	USA	Groundwater	380	Kim et al., 2007
Diclofenac	Canada	STP Effluent	70-250	Lee et al., 2005
	France	Mediterranean Sea	1500	Togola and Budzinki, 2008
	Germany (Berlin)	STP Effluent	2510	Heberer and Reddensen, 2001
	Germany (Berlin)	STP Effluent	100-131	Fang et al., 2012
	Germany (Berlin)	Groundwater	380	Heberer et al., 1997
	Germany (Frankfurt)	STP Effluent	810-2100	Ternes, 1998
	Greece	STP Effluent	890	Andreozzi et al., 2003
	Ireland	Atlantic Ocean	220-550	McEneff et al., 2014
	Luxembourg	STP Effluent	0.3-78	Pailler et al., 2009
	Portugal	Arade Estuary	31	Gonzalez Rey et al., 2015
	Spain	STP Effluent	140-2200	Gomez et al., 2007
	Spain	Turia river	49	Carmona et al., 2014
	Spain	Mediterranean Sea	4	Gros et al., 2012
	Spain	Mediterranean Sea	nd-<LOQ	Rodriguez-Navas et al., 2013
	Singapore	Indian Ocean	<1.5-11.6	Bayen et al., 2013
	Singapore	Indian Ocean	4-38	Wu et al., 2010
	Sweden	STP Effluent	700	Zorita et al., 2009

	Sweden	STP Effluent	120	Bendz et al., 2005
	Sweden	Hospital Effluent	380	Zorita et al., 2009
	Switzerland	STP Effluent	1300-2400	Tauxe-Wuersch et al., 2005
	Taiwan	STP Effluent	4-101	Lin and Tsai, 2009
	Taiwan	Indian Ocean	<2.5-53.6	Fang et al., 2012
	United Kingdom	Rivers	<20-599	Ashton et al., 2004
	United Kingdom	Estuaries	<8-195	Thomas and Hilton 2004
	United Kingdom	North Sea	<0.12	Nebot et al., 2007
Ibuprofen	Canada	Detroit River	141	Metcalfe et al., 2003
	Canada	STP Effluent	110-2170	Lee et al., 2005
	China	Yellow River	41	Wang et al., 2010
	China	Hai River	75	Wang et al., 2010
	China	Liao River	7	Wang et al., 2010
	China	Pear River	78	Wang et al., 2010
	France	Mediterranean Sea	1500	Togola and Budzinki, 2008
	Germany	STP Effluent	370-3400	Ternes, 1998
	Italy	Po River	4-1000	Castiglioni et al., 2005 Zuccato et al., 2006
	Italy	Adriatic Sea	<0.049-1.146	Loos et al., 2013
	Luxembourg	STP Effluent	3-359	Pailler et al., 2009
	Norway	Norwegian Sea	<0.07-0.7	Weigel et al., 2004
	Portugal	Ave River	ND-362	Paiga et al., 2013
	Portugal	Aveiro Lagoon	242	Paiga et al., 2013
	Portugal	Douro River	ND-239	Paiga et al., 2013
	Portugal	Leça River	ND-265	Paiga et al., 2013
	Portugal	Lima River	42-739	Paiga et al., 2013
	Portugal	Minho River	204	Paiga et al., 2013
	Portugal	Tamega River	359	Paiga et al., 2013
	Portugal	Ulma River	173	Paiga et al., 2013
	Portugal	Arade Estuary	28	Gonzalez Rey et al., 2015

	Singapore	Indian Ocean	41-121	Wu et al., 2010
	Singapore	Seawater	<2.2-9.1	Bayen et al., 2013
	Spain	Rivers	25	Gros et al., 2006
	Spain	Drinking water	5	Gros et al., 2012
	Spain	STP Effluent	3812	Gros et al., 2012
	Spain	STP Effluent	240-28000	Gomez et al., 2007
	Spain	Turia river	830	Carmona et al., 2014
	Spain	Mediterranean Sea	16	Gros et al., 2012
	Sweden	Hospital Effluent	8800	Zorita et al., 2009
	Sweden	STP Effluent	150	Bendz et al., 2005
	Switzerland	Lake Greifenn	5,1	Buser et al., 1999
	Switzerland	Lake Baldegger	1.5-3.5	Buser et al., 1999
	Switzerland	Lake Pfaffiker	4	Buser et al., 1999
	Switzerland	Lake Zurich	3.3-4	Buser et al., 1999
	Switzerland	STP Effluent	81	Buser et al., 1999
	Switzerland	STP Effluent	100-1200	Tauxe-Wuersch et al., 2005
	Taiwan	Indian Ocean	<2.5-57.1	Fang et al., 2012
	United Kingdom	Rivers	432-4201	Ashton et al., 2004
	United Kingdom	STP Effluent	1979-4239	Roberts and Bersuder, 2006
	United Kingdom	North Sea	<0.52	Nebot et al., 2007
	United Kingdom	Estuaries	<8-928	Thomas and Hilton 2004
Ketoprofen	Canada	STP Effluent	40-90	Lee et al., 2005
	France	Mediterranean Sea	6000	Togola and Budzinki, 2008
	Germany	STP Effluent	200-380	Ternes, 1998
	Japan	STP Effluent	68-219	Nakada et al., 2006
	Spain	STP Effluent	560	Gros et al., 2012
	Spain	STP Effluent	<LOQ-1760	Santos et al., 2005
	Spain	Mediterranean Sea	<8	Gros et al., 2012
	Sweden	STP Effluent	330	Bendz et al., 2005

	Taiwan	Indian Ocean	<1.7-6.59	Fang et al., 2012
	USA	STP Effluent	23	Thomas and Foster, 2004
Nimesulide	Ireland	STP Effluent	<LOQ-3500	Lacey et al., 2012
	Portugal	STP Effluent	<LOQ	Salgado et al.,2010
	Italy	Po river basin	nd-0.15	Ferrari et al., 2011

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804

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Table 2. Concentration of AMP, DIC, IBU, KET and NIM in *M. galloprovincialis* exposed to 0.5µg/L for 14 days. Data are given in ng/g dry weight (mean values ± standard deviations, n=5). LOD= limit of detection (1 ng/g for AMP, 0.5 ng/g for IBU and KET)

		AMP	DIC	IBU	KET	NIM
Exposure	Control	<LOD	<LOD	<LOD	<LOD	<LOD
	Exposed	<LOD	4.75 ± 5.30	<LOD	<LOD	8.00 ± 3.99

Table 3. Quantitative Hazard Quotients (HQ) and assigned class of hazard after weighted elaboration of biomarker results in mussels exposed to AMP, DIC, IBU, KET and NIM.

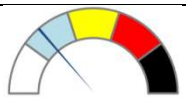
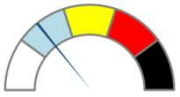
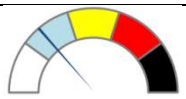
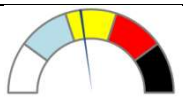
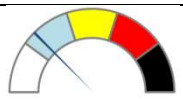
Treatment	HQ	Class of Hazard	Level
AMP	3.86	SLIGHT	
DIC	4.58	SLIGHT	
IBU	2.17	SLIGHT	
KET	18.69	MODERATE	
NIM	2.52	SLIGHT	

Table 4. Lists of the main biological processes modulated by KET and NIM exposures. The gene name of differentially expressed transcripts are reported for each process and treatment. ↓ and ↑ indicate down- and up-regulated genes.

	CTRL vs KET	**CTRL vs NIM**
METABOLISM		
Glycolysis / Gluconeogenesis	*↓GAPDH; ↑ PCK1, HK2, PCK2, PCXb*	*↓PGM1, TPI1A,GAPDH, ADH5; ↑ PCK1, PCK2, HK2*
Citrate cycle (TCA cycle)	*↓IDH3G, SUCLG1; ↑PCK1, PCK2, PCXB*	*↓IDH3G; ↑PCK1, PCK2*
Oxidative phosphorylation	*↓NDUFS2, ATP5G3B, ATP6V1D, NDUFV2, NDUFA6, MT-ND4; ↑ATP6V1F, NDUFA8, MT-ND5*	*↓COX4i1, NDUFA10, ATP6V1D, MT-CO1, ATP5G3, NDUFS2, NDUFV2, ATP6V0CB, ATP6V1E1B, ATP6V1H, NDUFA9; ↑MT-CO3*
Glycerolipid metabolism	*↓PLRP1, AK1BA*	*↓PLRP1, PPAP2A, GK2,AK1BA*
Fatty acid metabolism	*↓RNPEP, SC5D, ACOX1; ↑ ACAT1*	*↓RNPEP, ACAA2, SC5D, TPI1A, ADH5*
Drug metabolism	*↓XDH, TPMT, ↑ GSTA4, SULT1B1, GSTP2, HSP70I*	*↓ GSTM , GSTP1, UCK2A, MGST3, CYP1A, SULT1ST1, CYP3A65; ↑HSP70L, GMPS, IMPDH2*
TRANSCRIPTION/TRANSLATION/FOLDING/DEGRADATION		
Regulation of transcription	*↓BRF2, FOXG1A, ELF2A, HER6 TBX1, TAL1; ↑ NR1D2A,TBX20, MED17, KAT6A, CNOT3B, MAX, BARHL1A, SUPT5H, NR2E3, NR0B1, SETD1BA, GTF2H3*	*↓TRIM33, CBFB, LITAF, RARGB, CERS5, HER9, SUB1B, NR1D2B, HER6, FOSL1A; ↑SYNE1A, NR2E3, NR0B1, NFE2L2A, MAX, KDM2BA, SUPT5H, CHD4B*
Spliceosome	*↓SNRPE; ↑RBM25, HSPA8, SNRNP70, NCBP2,PRPF40A*	*↓TXNL4A, SNRPE, KCNF1B, SF3B14; ↑SF3B5,HSPA8, SNRNP70, PRPF40A, PUF60A, HNRNPM, CRNKL1*
Translation/ Aminoacyl-tRNA biosynthesis	*↑FARSB, RPSA, RPLP0, EIF5, RPL30, RSL24D1, HSP90AB, NARS*	*↓EEF1B2, EIF2S1B, RPL4; ↑FARSB, HARS, FARSA, RPLP2*
Protein folding	*↑DNAJB1B, HSP90AA1.1, DNAJA1, CCT6A, DNAJB5, PFDN2, HSP70L, HSP70B2, HSC70*	*↓TOR1L3; ↑PPIG, CLPXA, DNAJB1B, HSP90AA1.1, DNAJA2, DNAJA1, CCT7, TCP1, HSP90AA1.2, HSP70B2, , HSP70L,*
Proteolysis	*↓CTSC, RNPEP, ELA3L, PITRM1, ST14A, CTSL, CTSO, WWP2; ↑CUL1A, TCEB2, HERC1, VHLL, HUWE1, PIAS1B, UBE2L3A, CASP9*	*↓RFWD2, WWP2, FBXW8, , TRIM33, CTSC, CPVL, TLL1, CTSL, RNPEP, CTSL.1, PSMA2, CTSD, METAP1D, NAALAD2, UFSP2; ↑ADAM10A, PLG, BMP1A, BMP1B, HUWE1, TCEB1B, PIAS1B, TCEB2, SKP1*
DNA repair	*↓BABAM1; GADD45AB ↑ ERCC2, RAD1, GTF2H3, SESN1*	*↓MLH1; ↑RAD1, LIG1, PRP19, SESN1*

Table 4. continued

	CTRL vs KET	CTRL vs NIM
CELLULAR PROCESSES		
Lysosome	↓*LAPTM4A, AP3S2, CTSC, CTSO, CTSL*	↓*ATP6V1H, LAPTM4A, CTSL, CTSC, ATP6V0CB, CTSF, AP3S2, SLC17A5, CTSD, AP2B1, AP1S2, SMPD1, DNASE2B, AP5M1*
Apoptosis	↓*GABARAP, CASP2, CASP3A, CASP7* ↑*CASP9, BCL2L1, BIRC2, BAG4, RBM25, XIAP,*	↓*GABARAP, CASP3A, CASP3B, APIP, CASP2;* ↑*BAG4, BCL2L1, BIRC2, XIAP, BCL2*
Cell cycle	↓ *GADD45AB;* ↑*HSP70L, CDK6, MCM7, CDC26, SESN1, NUSAP1, SESN3*	↓*TXNL4A, MLH1;* ↑*HSP70L, SKP1, MNAT1, KATNA1, NUSAP1, SESN1, CHAF1B, SESN3, MND1, MAPK7*
PPAR SIGNALING PATHWAY		
PPAR signaling pathway	↓*DBI, ACOX1;* ↑ *UBB, PCK1, PCK2,LPIN1*	↓*GK2, DBI;* ↑*PCK1, PCK2, UBC, LPIN1*
IMMUNE SYSTEM		
Immune response	↓ *POLR3F, MGL* ↑*HLEC, CLEC4F, CLEC4G, CLEC4M, TLR1, TLR2, TLR6, MARCO*	↓*PGLYRP5, PGLYRP6, POLR3F, MGL* ↑*ECSIT, TLR1, TLR3, CLEC4F, CLEC4G,RBL*
ARACHIDONIC ACID		
Arachidonic acid metabolism	↓ *TBXAS1, PGR1, PTGER4, RNPEPL1, CYP4* ↑*PLA2*	↓*TBXAS1, PGR1, PTGER4, RNPEPL1, CYP4* ↑*PLA2*

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Legends of Figures

Figure 1. Biomarker responses in control mussels (CTRL) and in those exposed to AMP, DIC, IBU, KET and NIM. NRRT: Neutral Red Retention Time; G/H: granulocytes-hyalinocytes ratio; percentage of phagocytosis; LIPO: lipofuscin; NL: neutral lipids; ACOX: Acyl-CoA oxidase; AChE; acetylcholinesterase. Results are given as mean values ± standard deviations (n=5). Different letters indicate significant differences between groups of means (Newman-Keuls post hoc comparisons).

Figure 2. Antioxidant variations in control mussels (CTRL) and in those exposed to AMP, DIC, IBU, KET and NIM. CAT: catalase; GST: glutathione S-transferases; GPx H_2O_2 and GPx CHP: Se-dependent and sum of Se- dependent and Se-independent glutathione peroxidases; GR: glutathione reductase; GSH: levels of total glutathione; TOSC: total oxyradical scavenging capacity toward peroxyl (ROO•) and hydroxyl (HO•). Results are given as mean values ± standard deviations (n=5).

Figure 3. Biomarkers of genotoxic damage: percentage of DNA fragmentation and frequency of micronuclei (MN in control mussels (CTRL) and in those exposed to AMP, DIC, IBU, KET and NIM. Results are given as mean values ± standard deviations (n=5). Different letters indicate significant differences between groups of means (Newman-Keuls post hoc comparisons).

Figure 4. Principal Component Analysis (PCA) on biomarker data in control mussels (CTRL) and in those exposed to AMP, DIC, IBU, KET and NIM. Lipofuscin (LIPO), acetylcholinesterase activity (AchE), glutathione-S-transferases activity (GST), total glutathione (GSH), granulocytes-hyalinocytes ratio (G/H), catalase activity (CAT), Se-dependent and Se-independent glutathione peroxidases (GPx CHP), total Oxyradical Scavenging Capacity toward peroxyl radicals (TOSC ROO•), micronuclei frequency (MN) and DNA fragmentation.

Figure 5. Venn diagrams representing differentially expressed transcripts (total, down-regulated and up-regulated) in the comparisons CTRL vs KET and CTRL vs NIM exposed mussels.

Figure 6. Principal Component Analysis (PCA) on transcriptional results in control mussels (CTRL) and in those exposed to KET and NIM. The analysis was performed considering the entire set of probes (6A); the 1,154 differentially expressed genes (DEGs) observed in KET exposed mussels (6B); the 1,516 DEGs observed in NIM exposed mussels (6C).

Figure(s)

FIGURE 1

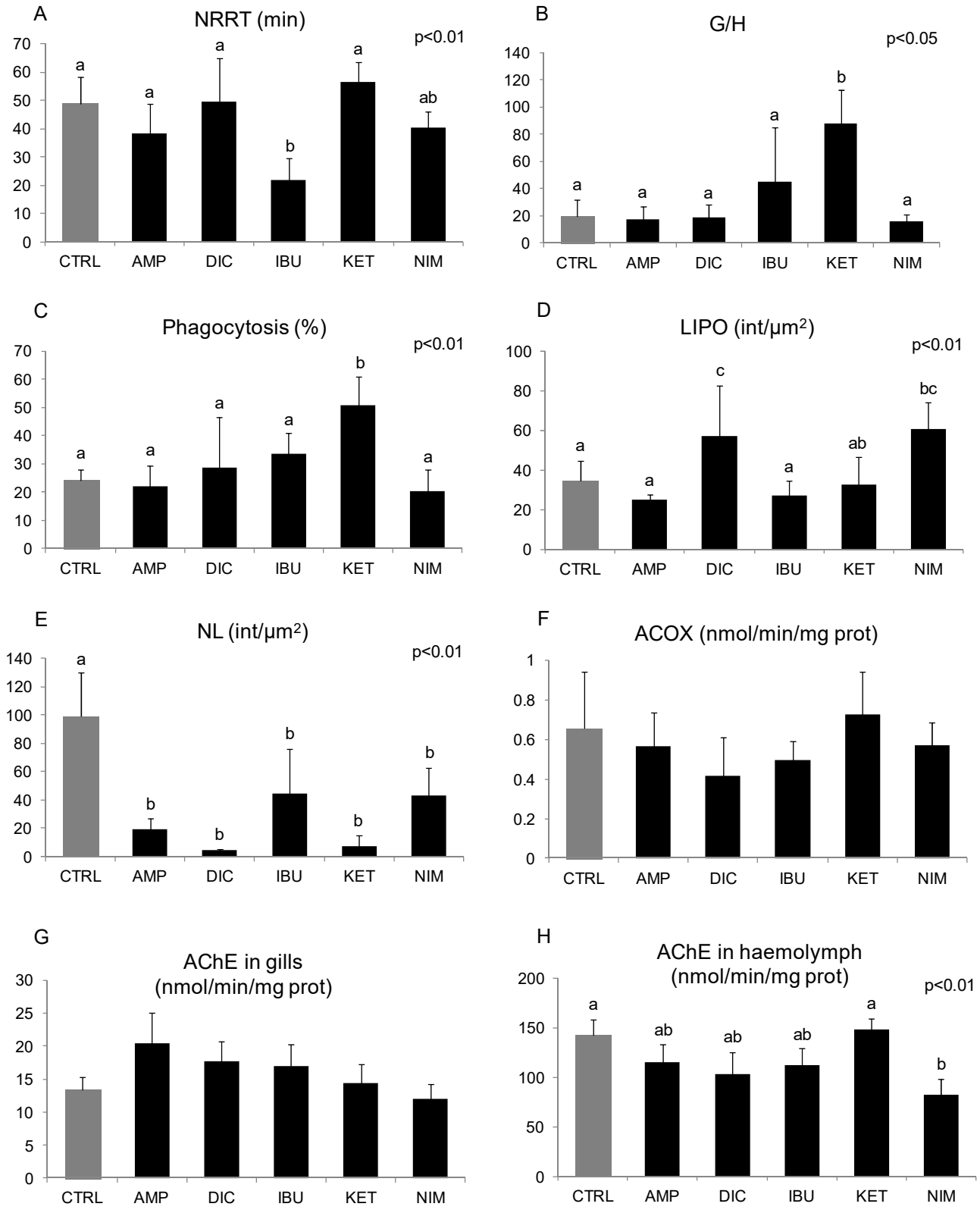
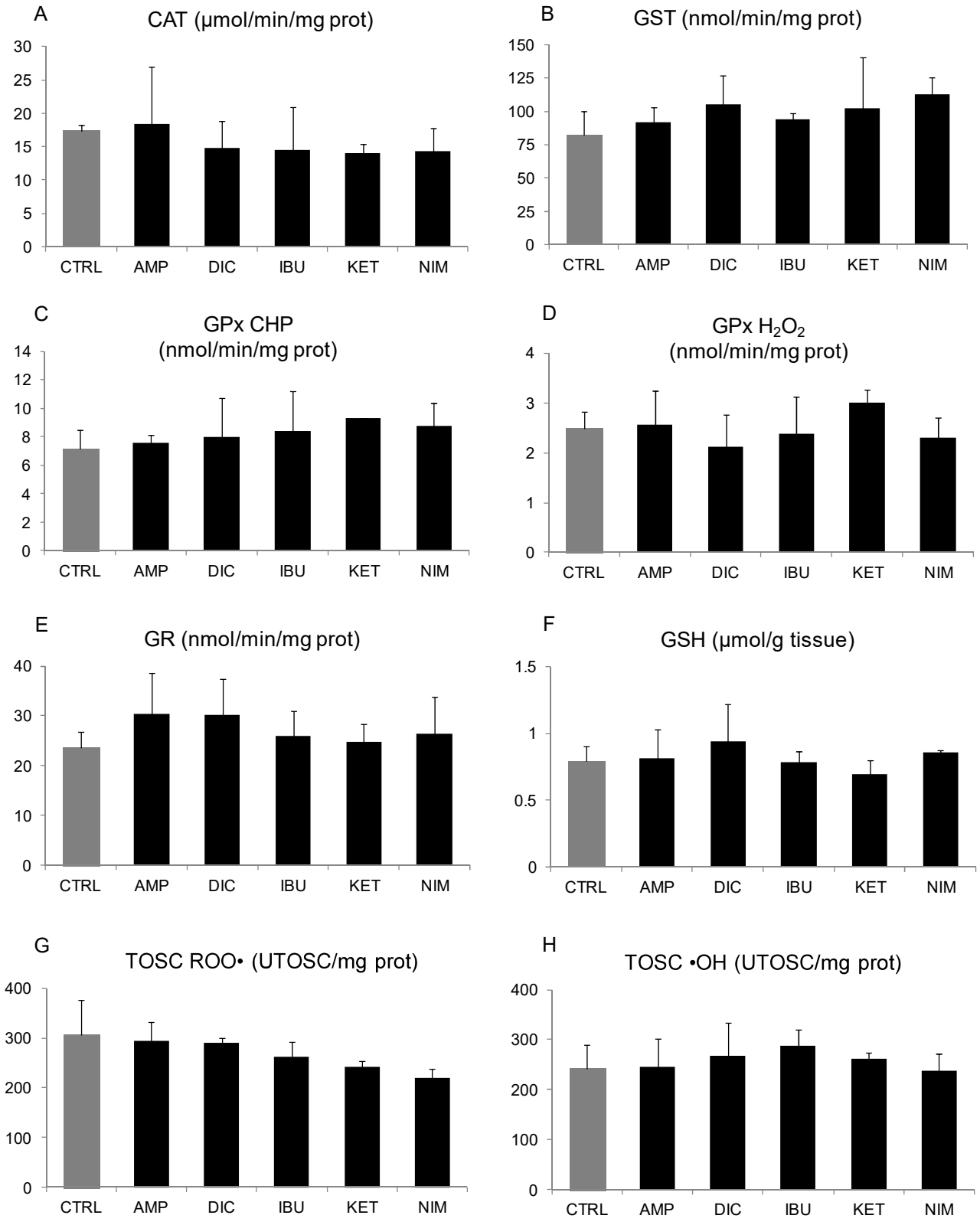


FIGURE 2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CAT (µmol/min/mg prot)
30
25
20
15
10
5
0
CTRL AMP DIC IBU KET NIM
B
GST (nmol/min/mg prot)
150
125
100
75
50
25
0
CTRL AMP DIC IBU KET NIM
C
GPx CHP
(nmol/min/mg prot)
14
12
10
8
6
4
2
0
CTRL AMP DIC IBU KET NIM
D
GPx H_2O_2
(nmol/min/mg prot)
4
3
2
1
0
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E
GR (nmol/min/mg prot)
40
30
20
10
0
CTRL AMP DIC IBU KET NIM
F
GSH (µmol/g tissue)
1.5
1
0.5
0
CTRL AMP DIC IBU KET NIM
G
TOSC ROO• (UTOSC/mg prot)
400
300
200
100
0
CTRL AMP DIC IBU KET NIM
H
TOSC •OH (UTOSC/mg prot)
400
300
200
100
0
CTRL AMP DIC IBU KET NIM

FIGURE 3

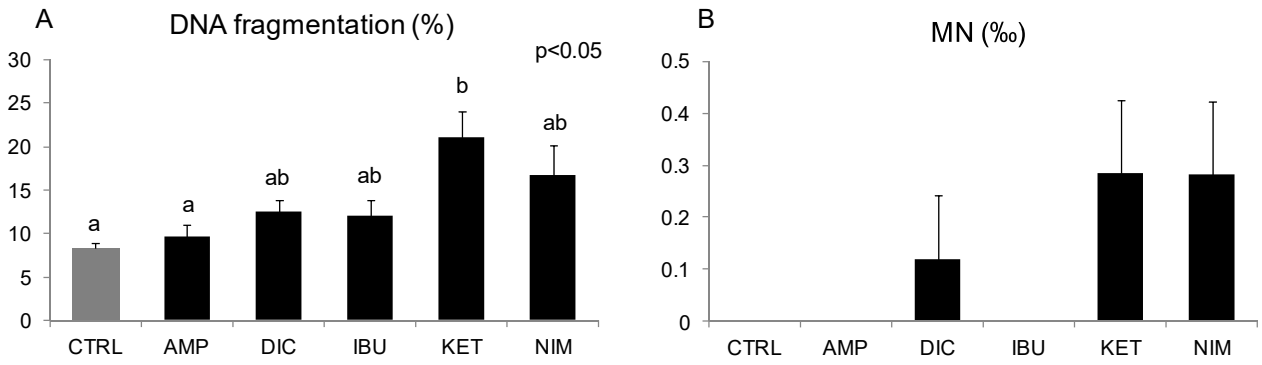

A
DNA fragmentation (%)
p<0.05
30
25
20
15
10
5
0
a
a
ab
ab
b
ab
CTRL
AMP
DIC
IBU
KET
NIM
B
MN (‰)
0.5
0.4
0.3
0.2
0.1
0
CTRL
AMP
DIC
IBU
KET
NIM

FIGURE 4

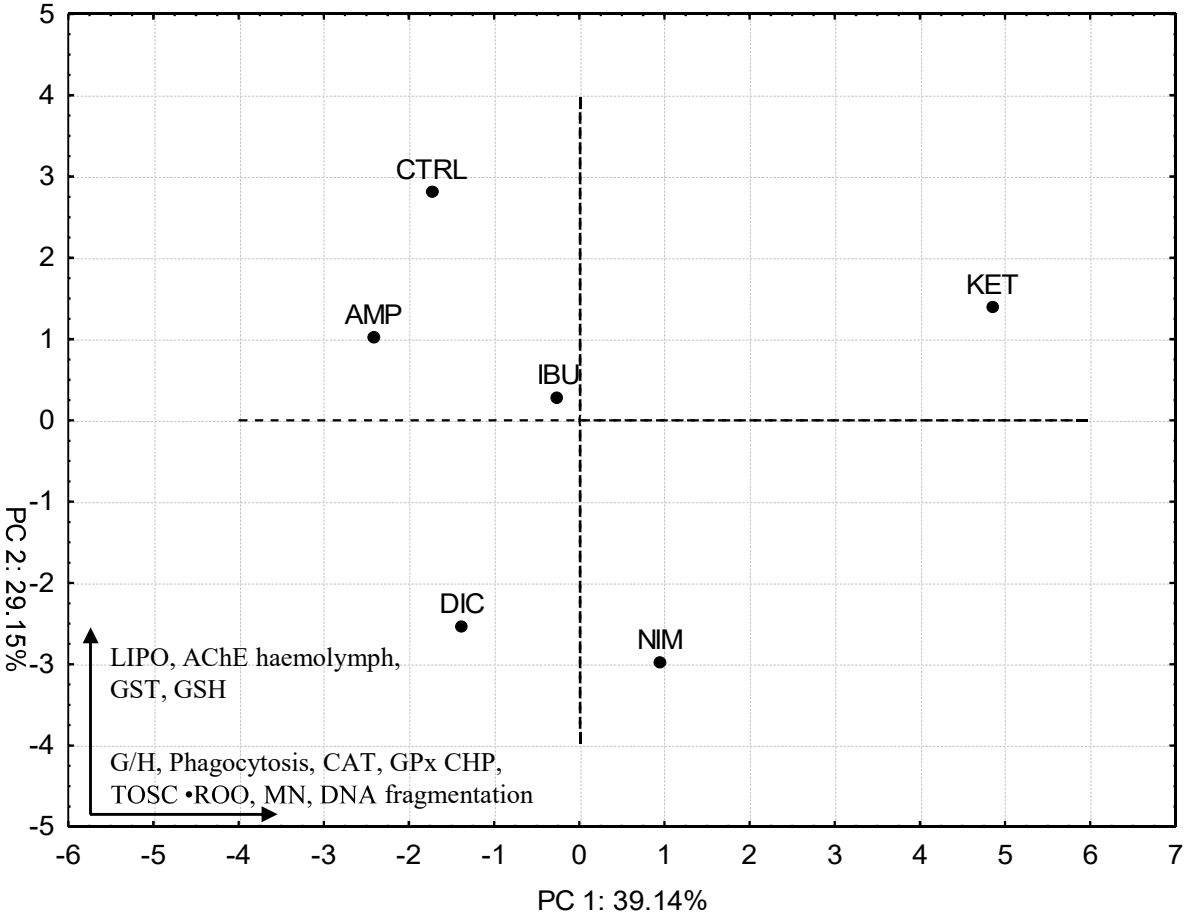

CTRL
AMP
IBU
KET
DIC
NIM
LIPO, AChE haemolymph,
GST, GSH
G/H, Phagocytosis, CAT, GPx CHP,
TOSC •ROO, MN, DNA fragmentation
PC 2: 29.15%
PC 1: 39.14%

FIGURE 5

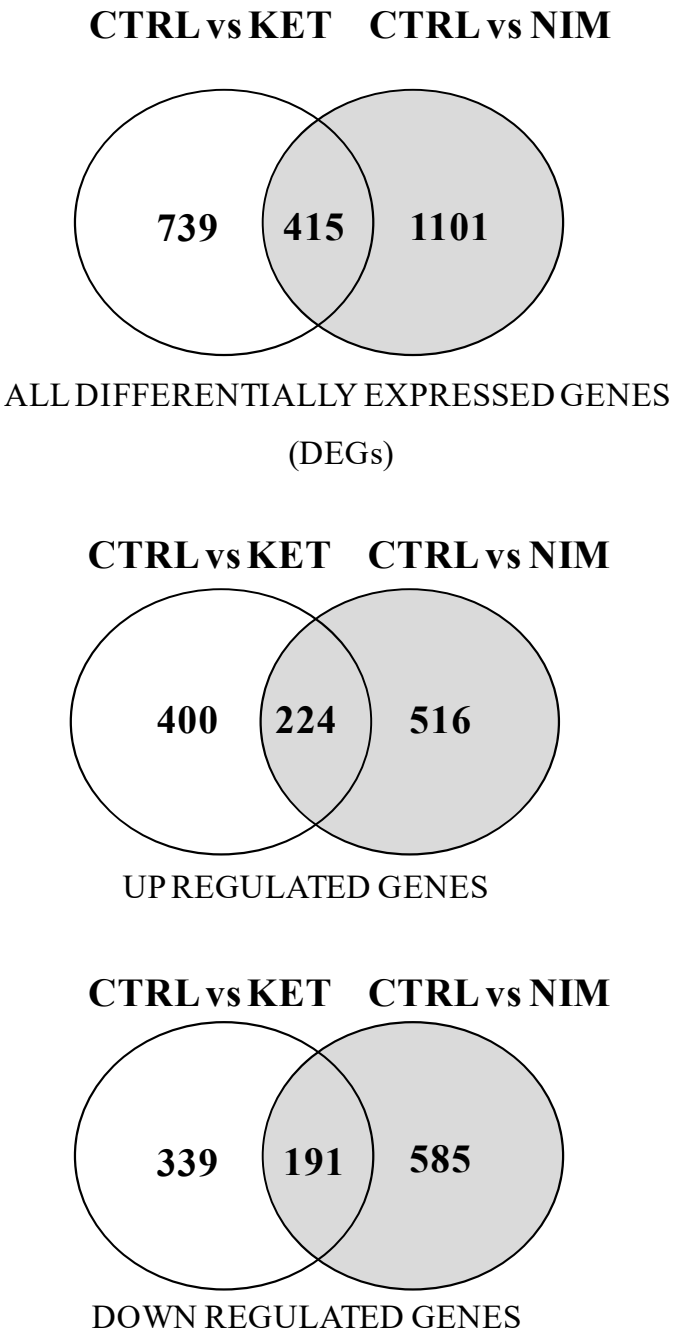
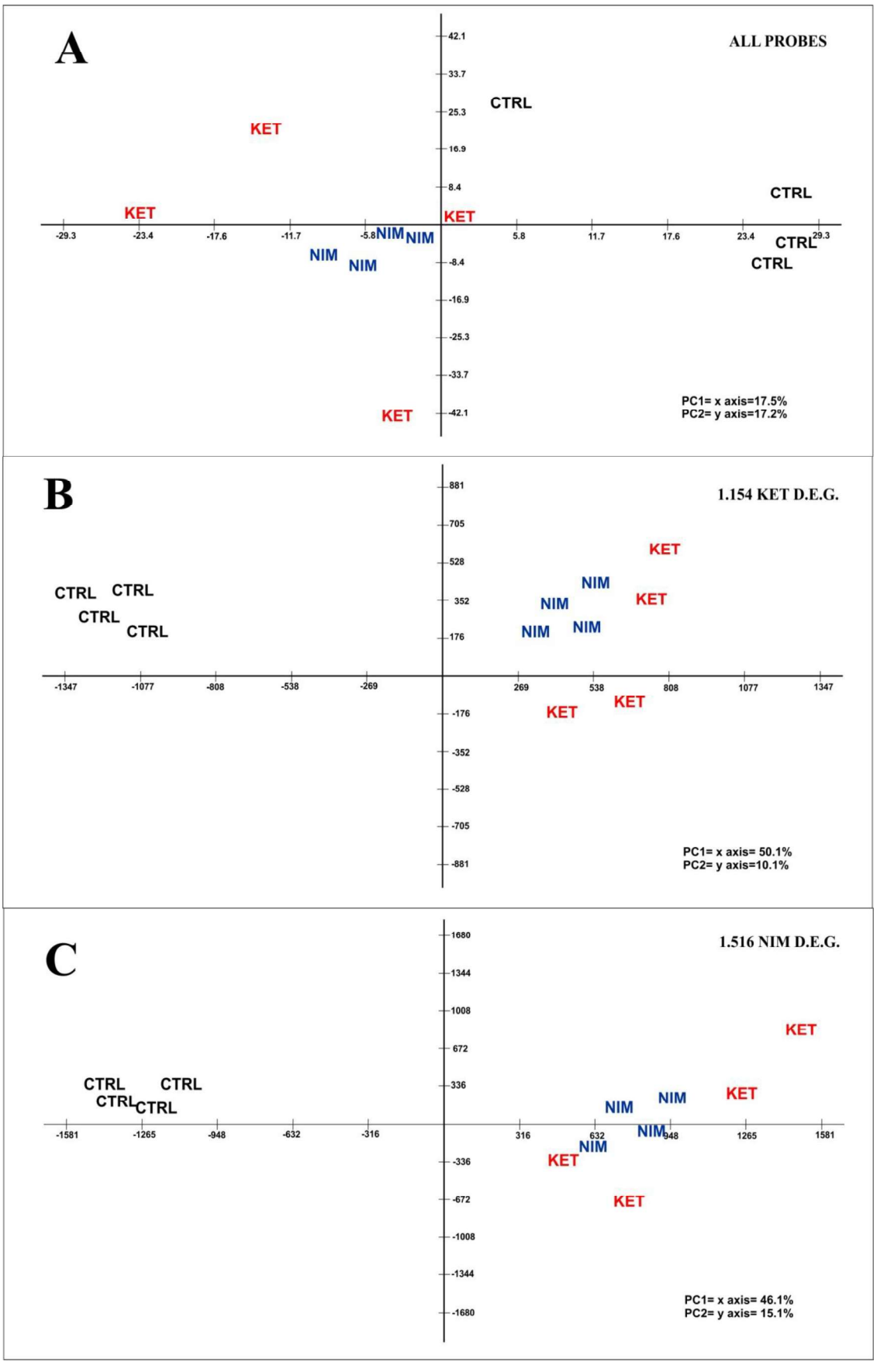

CTRL vs KET
CTRL vs NIM
739
415
1101
ALL DIFFERENTIALLY EXPRESSED GENES
(DEGs)
CTRL vs KET
CTRL vs NIM
400
224
516
UP REGULATED GENES
CTRL vs KET
CTRL vs NIM
339
191
585
DOWN REGULATED GENES

FIGURE 6


A
ALL PROBES
CTRL
KET
KET
CTRL
KET
NIM
NIM
NIM
NIM
CTRL
CTRL
KET
PC1= x axis=17.5%
PC2= y axis=17.2%
B
1.154 KET D.E.G.
CTRL
CTRL
CTRL
CTRL
KET
NIM
NIM
KET
NIM
NIM
KET
KET
PC1= x axis= 50.1%
PC2= y axis=10.1%
C
1.516 NIM D.E.G.
CTRL
CTRL
CTRL
CTRL
KET
NIM
NIM
KET
NIM
NIM
KET
KET
PC1= x axis= 46.1%
PC2= y axis= 15.1%

Supplementary Material 1

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HIGHLIGHTS

Transcriptional and cellular effects of NSAIDs were investigated in mussels

Biological effects occurred even with limited or absent accumulation of NSAIDs

NSAIDs caused alterations of immune responses, lipid metabolism, DNA integrity

DNA microarrays revealed molecular pathways that supported biomarker results

Transcriptional changes were more sensitive exposure biomarkers than cellular effects