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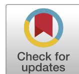

Environmental pharmaceuticals and climate change: The case study of carbamazepine in *M. galloprovincialis* under ocean acidification scenario

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ABSTRACT

Contaminants of emerging concern and ocean changes are key environmental stressors for marine species with possibly synergistic, but still unexplored, deleterious effects. In the present study the influence of a simulated ocean acidification scenario (pH = 7.6) was investigated on metabolism and sub-lethal effects of carbamazepine, CBZ (1 μg/L), chosen as one of the most widely diffused pharmaceuticals in marine organisms. A multidisciplinary approach was applied on mussels, *M. galloprovincialis,* integrating measurement of drug bioaccumulation with changes in the whole transcriptome, responsiveness of various biochemical and cellular biomarkers including immunological parameters, lipid and oxidative metabolism, onset of genotoxic effects. Chemical analyses revealed a limited influence of hypercapnia on accumulation and excretion of CBZ, while a complex network of biological responses was observed in gene expression profile and functional changes at cellular level. The modulation of gamma-aminobutyric acid (GABA) pathway suggested similarities with the Mechanism of Action known for vertebrates: immune responses, cellular homeostasis and oxidative system represented the processes targeted by combined stressors. The overall elaboration of results through a quantitative Weight of Evidence model, revealed clearly increased cellular hazard due to interactions of CBZ with acidification compared to single stressors.

1. Introduction

Challenges for the marine ecosystems have markedly evolved in the last decades in terms of typology of hazards, magnitude and frequency of disturbances. Beside traditional chemicals, contaminants of emerging concern like pharmaceuticals, endocrine disruptors, and microplastics are well recognized environmental stressors (Almeida et al., 2018; Avio et al., 2015; Heye et al., 2019; Mezzelani et al., 2018b). The ubiquitous presence of pharmaceuticals in freshwater and coastal ecosystems is caused by the massive use and often limited removal of those compounds by wastewater treatment plants (WWTPs). Potential bioaccumulation has been shown in aquatic species, with deleterious outcomes highlighted from molecular up to physiological levels in non-target marine organisms, and predictable adverse effects on development and reproductive success (Álvarez-Muñoz et al., 2015; Klosterhaus et al., 2013; Martínez Bueno et al., 2013; Mezzelani et al., 2016a, 2016b; Miller et al., 2019; Wille et al., 2011). Among human pharmaceuticals, the antiepileptic carbamazepine (CBZ) has been frequently measured in tissues of aquatic invertebrates: in Mediterranean mussels, *Mytilus galloprovincialis,* collected along the Italian coasts, more than 90% of the analyzed samples contained detectable levels of this active pharmaceutical ingredient with concentrations up to 300 ng/g dry weight (Mezzelani et al., 2020). The onset of oxidative stress, lipid peroxidation, impairment of immune system and genotoxic damage were documented in marine invertebrates exposed to a wide range of environmentally realistic concentrations of CBZ (0.3–3.0 and 6.0–9.0 μg/L) (Almeida et al., 2014, 2015, 2017; Freitas et al., 2016).

Scientific effort has been dedicated to investigate the biological

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impact of individual pharmaceuticals and, in a few cases, of mixtures (Franzellitti et al., 2019; Mezzelani et al., 2018a; Trombini et al., 2019). Considering the multitude of environmental stressors to which organisms are simultaneously exposed, further research is urgently needed to elucidate also the role that future projections of temperature and CO_2-driven ocean changes will have on environmental fate, bioavailability and toxicity of such emerging compounds (Delorenzo, 2015). The reduction of seawater pH has both direct effects on several metabolic pathways in marine organisms, as immunocompetence, oxidative status, growth and reproduction (Gazeau et al., 2013; Kroeker et al., 2010), and it also indirectly modulate bioaccumulation and biological responsiveness to other contaminants (Cao et al., 2019, 2018; Giuliani et al., 2020; Götze et al., 2014; Hawkins and Sokolova, 2017; Munari et al., 2019; Nardi et al., 2018a, 2018b). Recent studies on the interaction between ocean acidification (OA) and pharmaceuticals revealed a synergistic increase of CBZ toxicity (3 μg/L) in *Scrobicularia plana* at pH 7.1, with reduced specimens survival and enhanced oxidative stress (Freitas et al., 2016). At a lower CBZ dose (1 μg/L) and pH of 7.5, *Ruditapes philippinarum* exhibited changes in mRNA transcription of several genes related to neurotransmission, immunity and biomineralization (Almeida et al., 2018).

However, mechanistic pathways of interactions between different typologies of multiple stressors have been scarcely investigated. The urgency to explore the ecotoxicological potential of compounds of emerging concern (like CBZ) under expected climate changes scenarios is emphasized by the European Commission (EC) within the “Strategic Approach to Pharmaceuticals in the Environment” (COM/2019/128 final). Understanding long term consequences is an important prerequisite also promoted by the 14th of the 17 Sustainable Development Goals (SDGs) of the 2030 Agenda for Sustainable Development.

In this respect, the present study was aimed to investigate the influence of reduced pH on bioaccumulation, excretion and sub-lethal effects of CBZ in *M. galloprovincialis.* A multidisciplinary approach was applied combining measurement of drug bioaccumulation, transcriptomic responses through RNA-sequencing and a large number of biomarkers reflecting the perturbation of different cellular districts and biochemical pathways: such responses included alteration of immunological parameters, lipid and oxidative metabolism, onset of genotoxic effects. The integration of transcriptomic analyses with functional effects at cellular level was expected to clarify the activation mechanisms of molecular responses and potentially elucidate the intricate Mechanism of Action (MoA) of CBZ in marine mussels. The overall results were elaborated to summarize a cellular hazard index based on toxicological relevance, magnitude of variations and responsiveness of analyzed endpoints. The present study was expected to provide novel insights on the interactive effects of pollutants of worldwide emerging concern with ocean acidification, thus representing a further step to clarify the environmental risk of multiple stressors.

2. Materials and methods

2.1. Animal collection and experimental design

Mussels, *M. galloprovincialis* (6.0 ± 0.5 cm shell length), were obtained in March 2019 from a shellfish farm in an unpolluted area of Central Adriatic Sea (Regoli et al., 2014) and acclimatized for 10 days in eight 20 L aquaria (57 organisms per tank) with aerated artificial seawater (ASW) at local seasonal environmental conditions of salinity (35 practical salinity units), temperature (13 °C) and pH (8.10). Collection and experimental use of these organisms is not subjected to ethical review permissions according to both European and Italian normative (Directive 2010/63/EU, 2010; Italian Legislative Decree n. 26, 4/03/2014), while monitoring guidelines recommend this organism as appropriate bioindicator organism for assessing bioavailability of ecotoxicological effects of environmental pollutants in marine environments (Bocchetti and Regoli, 2006).

At the end of acclimation, mussels were exposed to clean or carbamazepine-contaminated seawater under environmental or reduced-pH conditions, according to the following experimental treatments, each performed in duplicate tanks: control (CTL): pH = 8.10/pCO_2= ~400 μatm; exposure to carbamazepine (CBZ): 1 μg/L CBZ, pH = 8.10/pCO_2= ~400 μatm; reduced pH/hypercapnia (ACD): pH = 7.6/pCO_2= ~1700 μatm; ACD + CBZ: pH = 7.6/pCO_2= ~1700 μatm, CBZ 1 μg/L. After 28 days of exposure, organisms were maintained for additional 10 days in carbamazepine-free ASW at respective pH used during exposure to investigate depuration kinetics.

The exposure dose of CBZ can be found in coastal areas (Birch et al., 2015; Freitas et al., 2016; Gaw et al., 2014; Mezzelani et al., 2018b), while target pH was selected following predicted condition for the end of the century based on CO_2 emissions scenario RCP 8.5 (Hoegh-Guldberg et al., 2015). A stock solution of carbamazepine (Sigma Aldrich) was prepared in methanol and stored at room temperature for the duration of the experiment, while working solutions were prepared daily by diluting the stock solution in ASW: during exposures, water was changed every other day and carbamazepine re-dosed. Reduced pH/hypercapnia conditions were obtained by bubbling pure CO_2 in ASW until the desired pH was reached; this condition was monitored and maintained in exposure tanks through pH-stat system (Aquatronica®). For each experimental condition pH was measured continuously, temperature and salinity daily, while total alkalinity (A_T) twice per week according to Dickson et al., 2007. Seawater carbonate parameters (pCO_2 and saturation state (Ω) for calcite and aragonite) were calculated in CO2SYS (Pierrot et al., 2006) using barometric pressure values, as well as A_T, pH, temperature and salinity values of the respective samples; full seawater chemistry is provided in Supplementary Materials (SM1 and Table SMT1), while details of constants used for calculations can be found in Nardi et al., (2017). Mussels were fed 12 h prior the water change with a commercial mixture of zooplankton (50–300 μm) for filter-feeding organisms: zooplankton was preferred to live phytoplankton to avoid any possible interference on the inorganic carbon system. Samples for chemical analyses of accumulated carbamazepine were collected at day 0, 3, 7, 14, 21, 28, 31 and 38, while those for molecular, biochemical and cellular alterations were collected at the end of CBZ-exposure (day 28). For chemical analyses, 3 pools each constituted by the whole tissues of 2 organisms were collected at each time and stored at −20 °C. For transcriptomic, biochemical and histochemical analyses, gills, digestive glands and hemolymph were collected from 24 individuals, pooled in 8 separate samples, each constituted by tissues of 3 individuals, rapidly frozen in liquid nitrogen and maintained at −80 °C. At the same time, aliquots of hemolymph were immediately processed for *in vivo* analysis of hemocytes lysosomal membrane stability, granulocytes-hyalinocytes ratio, phagocytosis rate and DNA damage; additional aliquots of hemolymph were fixed in Carnoy’s solution (3:1 methanol, acetic acid) for the evaluation of micronuclei frequency.

2.2. Chemical analyses

Concentrations of CBZ in mussel tissues were determined by High Performance Liquid Chromatography with fluorimetric and diode array detectors. Information on reagents, as well as detailed extraction and analytical protocols, including QA/QC procedures, are given in SM1.

2.3. Biomarkers analyses

Validated protocols were used to analyze the following classes and typologies of biomarkers (SM1): immunological responses (lysosomal membrane stability LMS, phagocytosis rate, granulocytes/hyalinocytes ratio G/H), genotoxic damage (loss of DNA integrity and micronuclei frequency, MN) and neurotoxic effects (acetylcholinesterase AChE in hemocytes); peroxisomal proliferation (Acyl-CoA oxidase ACOX), single antioxidant defenses (catalase CAT, glutathione S-transferases GST, Se-dependent glutathione peroxidases Se-dep. GPx, total GPx, glutathione

reductase GR, total glutathione TGSH), total antioxidant capacity (total oxyradical scavenging capacity TOSC toward peroxyl radical ROO•, hydroxyl radical HO• and peroxynitrite $ONOO^-$) and lipid peroxidation products (malondialdehyde MDA) in digestive gland. In addition, cryostat sections of digestive glands were analyzed for lipofuscin and neutral lipids content.

2.4. Transcriptomic analysis: RNA extraction, RNAseq library preparation and sequencing

Total RNA was extracted from 5 pools of mussel digestive glands (tissues of 5 specimens for pool) per each experimental group (CTL, CBZ, ACD, ACD + CBZ):RNeasy Mini Kit (Qiagen, Hilden, Germany) was used following the manufacturer's protocol with additional DNAse treatment (Qiagen). RNA purity, concentration and integrity of each pool were checked using Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA Integrity Number (RIN) index was calculated using Agilent 2100 Expert software. Sure Select Strand-Specific mRNA Library (AgilentTechnologies, Santa Clara, CA, USA) was used for the libraries construction according to the manufacturer's protocol (see Milan et al., 2018a). Briefly, the first step of RNA library preparation method was the purification of poly(A) RNA from total RNA using two serial rounds of binding to oligo(dT) magnetic particles. After the chemical fragmentation of poly(A) RNA, single-stranding cDNA, the double-stranding cDNA was obtained through the Adenylate cDNA'3-ends and the ligate adaptors adding. The final step consisted in the adaptor-ligation cDNA library using the PCR amplification. The libraries were quantified with a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and the quality was assessed by Agilent 2100 Bioanalyzer. The library pools were sequenced on 2 lanes of a HighSeq 4000 (Illumina, Davis, CA, USA) with a single 1*100 bp setup, obtaining a total of 673, 705, 767 reads (sequences available in NCBI SRA; https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA611904).

2.5. Statistical analyses

Statistical analyses for CBZ bioaccumulation and biomarkers data were performed using RStudio (version 1.2.5033). Data were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test); when assumptions were not fulfilled, data were transformed using Box-Cox transformation (power transformation, R package "MASS"). Effect of the factors CBZ-exposure, pH, time of exposure and their interactions on CBZ bioaccumulation in mussels tissues were assessed using a generalized linear model (three-way ANOVA, SM1-Table SMT2) followed by Tukey HSD (HSD) *post-hoc* test for comparing the means of interest. Similarly, also for biomarkers (measured only at day 28), the effect of CBZ-exposure, pH and of their interaction were assessed using a generalized linear model (two-way ANOVA, SM1-Table SMT4), followed by Tukey HSD *post-hoc* tests. Multivariate principal component analysis (PCA) was applied to visualize the relationships among the different treatments.

For each treatment, the whole dataset of biomarkers results was summarized in a hazard index elaborated through weighted criteria which assign to each biomarker a "weight" based on its toxicological relevance and a "threshold" for the minimum change considered the biological significance (Sediqualsoft, Piva et al., 2011). Variations measured for each biomarker are thus normalized toward their specific thresholds and corrected for the weight and the statistical significance of the difference compared to controls (for full details see Regoli et al., 2019): the calculated Hazard Quotient (HQ) does not include biomarkers with variations lower or equal to their threshold, while it averages or adds the summation (Σ) respectively for those biomarkers with variations up to 2-fold or more than 2-fold greater than the specific threshold (Avio et al., 2015; Benedetti et al., 2012; Mezzelani et al., 2018a; Piva et al., 2011; Regoli et al., 2014). The model finally assigns the elaborated HQ to one of five classes of hazard, from Absent to Severe. Whole calculations and assumptions have been fully given elsewhere (Regoli et al., 2019).

For gene expression statistical analyses, raw reads were quality checked with FastQC/v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and subsequently adaptors and low-quality reads were trimmed using Trimmomatic/v0.36 (Bolger et al., 2014) "Single End (SE)" with default parameters. High-quality reads were subsequently mapped against the reference transcriptome from Moreira et al., 2015, by using Kallisto/v0.46.1 (Bray et al., 2016) with options "–single –fragment-length 100 –sd 40". The count table was generated with the script "abundance_estimates_to_matrix.pl" of the Trinity/v2.9.0 (Haas et al., 2013) suite and imported in R/v3.6.0. The count matrix was filtered to remove contigs with less than 5 reads in at least 4 biological samples to remove contigs with low coverage which would contribute to background noise (Peruzza et al., 2019). A total of 40,552 contigs were maintained for gene expression analyses. To normalize the count table and remove unwanted variation, the functions "betweenLaneNormalization" (with upper quartile method) and "RUVs" (with $k = 9$ factors) from the R package RUVSeq/v1.14.0 (Risso et al., 2014) were used and the Principal Component Analysis (PCA) was generated with the function "plotPCA". Differential expression (DE) analysis was performed by using a likelihood ratio test with the functions "glmFit" and "glmLRT" of the package edgeR/v3.22.5 (Robinson et al., 2010) on selected pair-wise comparisons: "CBZ vs CTL"; "ACD vs CTL"; "CBZ + ACD vs CTL" and genes were deemed as DE with a cut-off FDR < 0.05 and Fold change (FC) > 2. Subsequently, functional interpretation of differentially expressed genes was obtained by enrichment analysis using Database for Annotation, Visualization, and Integrated Discovery (david ver. 6.8) software (Dennis et al., 2003; Huang, Sherman, & Lempicki, 2009), considering GO Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) Database and KEGG pathways. DAVID retrieves the functional annotation of differentially expressed genes through enrichment analyses based on an integrated biological knowledge-base containing over 40 annotation categories. Since DAVID databases contain functional annotation data for a limited number of species, *M. galloprovincialis* transcripts were associated with Swissprot sequence identifiers. IDs corresponding to differentially expressed genes revealed in each treatment and to all genes represented in the *M. galloprovincialis* transcriptome (40,552) were obtained and were then used in DAVID to define a "gene list" and a "background," respectively. A functional annotation was obtained for significant genes identified by each pairwise comparison (CTL vs. CBZ; CTL vs. ACD; CTL vs. ACD + CBZ). DAVID settings: gene count = 2 and ease = 0.1. The same analyses have been performed considering separated up- and down-regulated genes within each treatment.

3. Results

Concentrations of CBZ in tissues of control and exposed mussels are reported in Fig. 1 and in SM1-Table SMT3, while three-way ANOVA results are given in SM1-Table SMT2. Values measured at the beginning of the experiment (day 0) were 37.7 ± 7.5 ng/g dry weight (d.w.) which generally decreased below the detection limit in non-CBZ exposed organisms during the experiment (SMT2). Mussels exposed to CBZ revealed a significant increase in tissue levels of this drug with a peak after 3 days and values always greater than in control groups for the whole duration of experiment. Concentrations markedly increased also in mussels exposed to CBZ at reduced pH with average values comparable to those observed in organisms exposed at control pH. After 10 days of depuration (day 38), levels of CBZ were similar to those observed at the beginning of the experiment. The overall results did not highlight a significant effect of reduced pH on CBZ bioaccumulation and depuration in exposed mussels.

Among immunological biomarkers, the lysosomal membrane stability, the phagocytosis capacity and granulocytes/hyalinocytes ratio

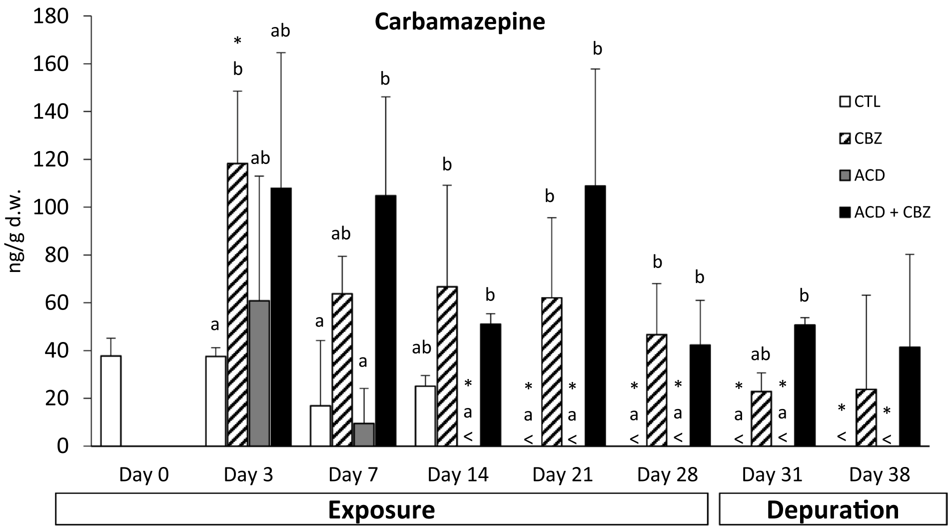

Fig. 1. Concentration at different sampling times of Carbamazepine in the whole tissues of M. galloprovincialis exposed to experimental conditions. Data are referred to both exposure and depuration phase, given in ng/g dry weight (mean values ± standard deviations, n = 3). Lower case letters are used to highlight significant differences between treatments within the same sampling time, asterisks (*) represent significant difference compared to T0 organisms, "<" indicates concentration below limit of detection, LOD (1.03 ng/g d.w).

were significantly reduced by CBZ, with no additional effect of pH (Fig. 2A-C, SM1-Table SMT4).

A significant induction was caused by CBZ and acidification on the acetylcholinesterase activity, with a greater effect of the drug when dosed alone (Fig. 2D, SM1-Table SMT4). Considering biomarkers of genotoxic damage, micronuclei frequency increased in all treatments, with a statistically significant effect of hypercapnic condition, either alone or in combination with CBZ (Fig. 2E, SM1-Table SMT4); no effect

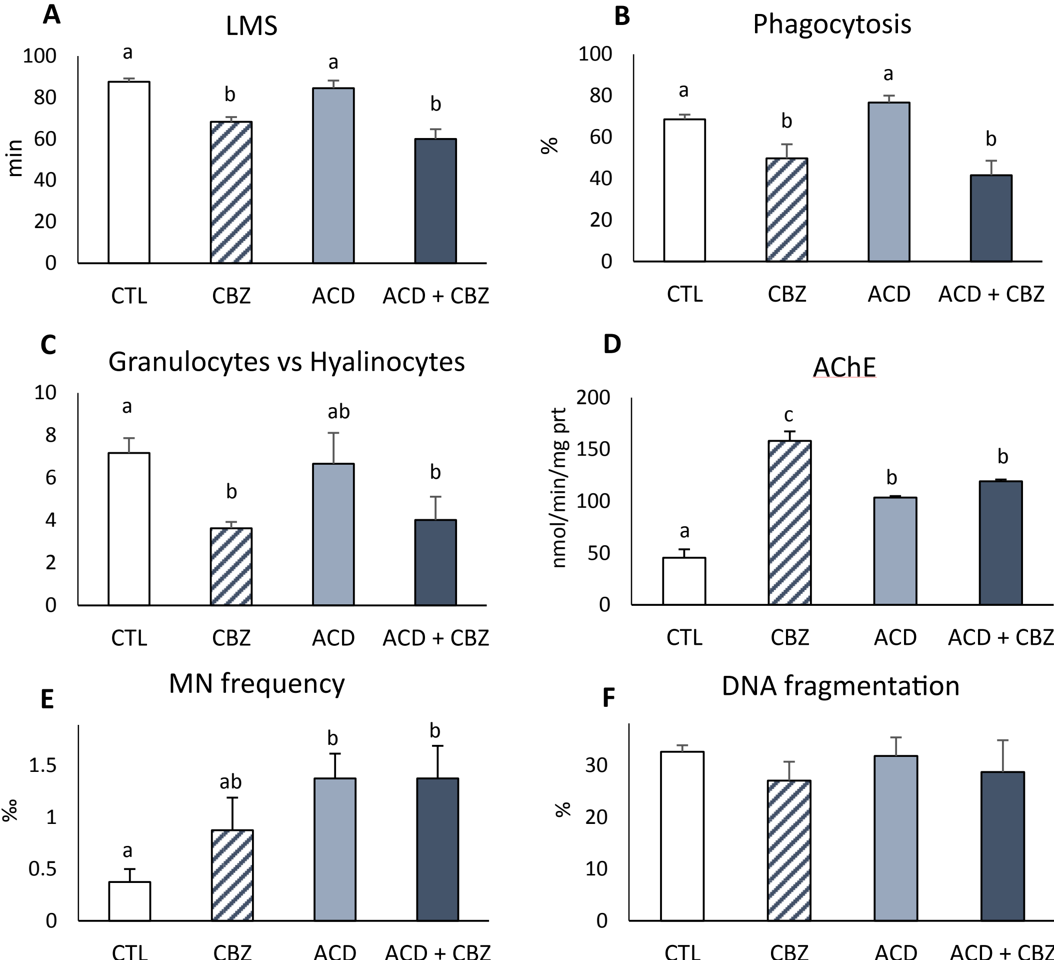

Fig. 2. Lysosomal membrane stability (A), phagocytosis rate (B), granulocytes/hyalinocytes ratio (C), Acetylcholinesterase activity (D), frequency of micronuclei (E) and DNA damage (F) in haemocytes of mussels exposed to various treatments. Data are given as mean values ± Standard Error of the Mean (n = 5). Different letters indicate significant differences between group of means, CTL Control; CBZ Carbamazepine; ACD acidification; ACD + CBZ acidification + CBZ.

was observed in terms of DNA fragmentation (Fig. 2F).

Both CBZ and hypercapnia condition significantly increased lipofuscin content in tertiary lysosomes, with cumulative effects observed in co-exposed organisms (Fig. 3A, SM1-Table SMT4). Neutral lipids were also enhanced by CBZ and hypercapnia, with significant interactions revealed between these two factors (Fig. 3B, SM1-Table SMT4). No variations were measured for MDA content and ACOX activity (Fig. 3C-D).

The antioxidant defenses showed few variations (Fig. 4A-I, SM1-Table SMT4). Among these, glutathione S-transferase was significantly increased by CBZ exposure and lowered by acidification, but these effects were not elicited by interaction of these factors; a significant interaction between CBZ and low pH was revealed by the different effects caused on Se-dependent glutathione peroxidases. A common trend, not statistically significant but worthy to note, was observed for mussels co-exposed to CBZ and low pH for catalase, total glutathione peroxidases and total oxyradical scavenging capacity (TOSC) toward hydroxyl radicals.

The principal component analysis on the whole dataset of biomarkers provided a two-dimensional pattern (Fig. 5), explaining almost 46% of the total variance. A clear separation was observed between control and treated organisms, while a further discrimination occurred between those treated with carbamazepine (alone and at low pH) and mussels exposed only to acidification. The parameters determining the separation along PC1 axis, were phagocytosis rate, lysosomal membrane stability, granulocytes vs hyalinocytes ratio, and lipofuscin content; along the PC2 axis, the separation was mostly determined by MN frequency, TOSC ROO•, TOSC HO• and TOSC ONOO–.

The overall biological significance of cellular responses observed in each experimental condition was summarized in a single hazard index through the application of weighted criteria which consider the toxicological relevance and magnitude of observed variations for all the biomarkers (Fig. 6). The elaborated hazard quotient (HQ) was “Slight” for organisms exposed to individual stressors (CBZ, ACD), while raised to “Moderate” after the co-exposure treatment, further supporting interactive effects of these factors on measured cellular responses.

Results on gene expression profiles were analyzed via Principal Component Analyses (PCA) considering a total of 40,552 contigs. The first axis explaining the 15.32% of the variation of gene expression profiles confirmed a clear separation of the control groups from all treatments, while the second component (13.53%) discriminated the three tested treatments (ACD, CBZ and ACD + CBZ) (Fig. 7). Pairwise comparisons between the CTL group and ACD, CBZ and ACD + CBZ revealed a total of 972, 697 and 666 DEGs (p-value $\leq$ 0.05, FC $>$ 2), respectively. In all pairwise comparisons, exposed mussels showed a higher number of over-expressed genes compared to CTL group (802, 542, and 512 up-regulated genes in ACD, CBZ and ACD + CBZ, respectively). The whole lists of DEGs and their putative annotation are reported in Supplementary Material 2, SM2. Of all the DEGs, nearly 6% (106 genes) was shared between the three exposed groups compared to control (Fig. 8), while the highest percentage of common DEGs among two treatments was found between ACD and ACD + CBZ exposed mussels (253 DEGs; 14.1%). Enrichment analyses through DAVID software were also carried out to obtain a functional annotation at each treatment, considering the full list of differentially expressed genes as well as up- and down-regulated genes separately (Supplementary Material 3, SM3).

CBZ exposed mussels showed the up-regulation of genes involved in drug metabolism such as *Cytochrome P450 4F8* (*CYP4F8), Cytochrome P450 3A2 (CYP3A2), Cytochrome P450 3A29 (CYP3A29)*) and *Sulfotransferase family cytosolic 1B member 1* (*ST1B1*), as well as the stress-induced proteins *Sestrin-3* (*SEST3*) that has a role in protection against oxidative and genotoxic stresses. Several genes of cell cycle (18 DEGs) and DNA repair (7 DEGs) were also differentially expressed (Table 1). Among the DEGs, *Sodium-dependent serotonin transporter* (*SC6A4*) was more than 60 times down-regulated in CBZ exposed mussels compared to CTL. An opposite trend has been revealed for the up-regulation of two genes belonging to GIMAP family (GIMAP4, GIMAP7) with a role in defense, cell differentiation and apoptosis, as well as for three genes coding for *Baculoviral IAP repeat-containing protein (BIRC1, BIRC7),* which modulate apoptotic processes. It should be also highlighted the significant up-regulation of several genes of immune response and inflammation. Enrichment analyses of DEGs in CBZ exposed mussels confirmed significant changes in xenobiotic metabolism by the enriched terms “response to chemical” (45 DEGs), “cellular response to chemical stimulus” (30 DEGs) and “response to organic cyclic compound” (SM3). Several terms related to synapses and neurotransmission were significantly enriched, such as the CC_terms “synapse” (17 DEGs) and “postsynapse (12 DEGs), and the BP_terms “regulation of synapse organization” (6DEGs) and “synapse assembly” (5DEGs), indicating

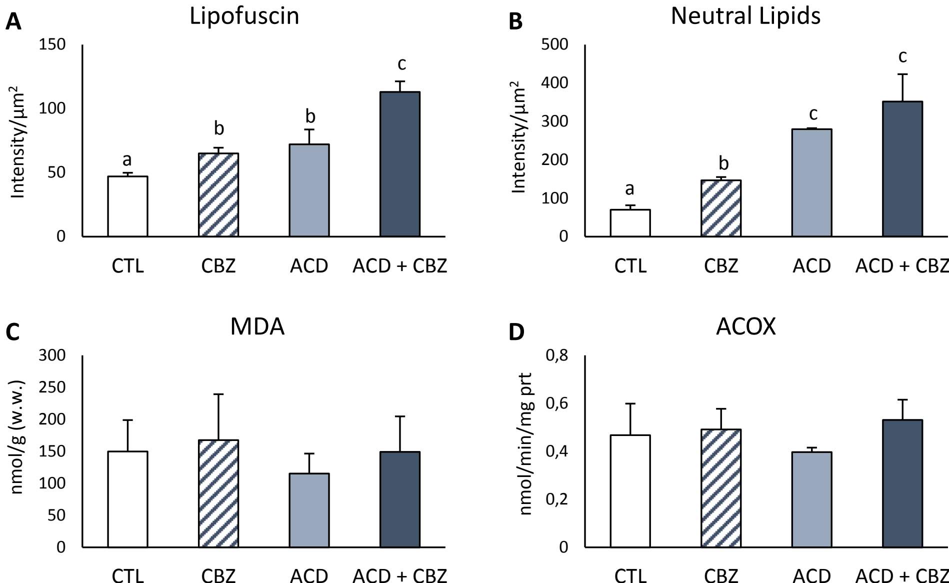

Fig. 3. Oxidative stress biomarkers in digestive gland of mussels exposed to various treatments. Lipofuscin (A), Neutral Lipids (B), MDA: levels of malondialdehyde (C), ACOX Acyl-CoA oxidase activity (D). Data are given as mean values ± Standard Error of the Mean (n = 5). Different letters indicate significant differences between group of means, CTL Control; CBZ Carbamazepine; ACD acidification; ACD + CBZ acidification + CBZ.

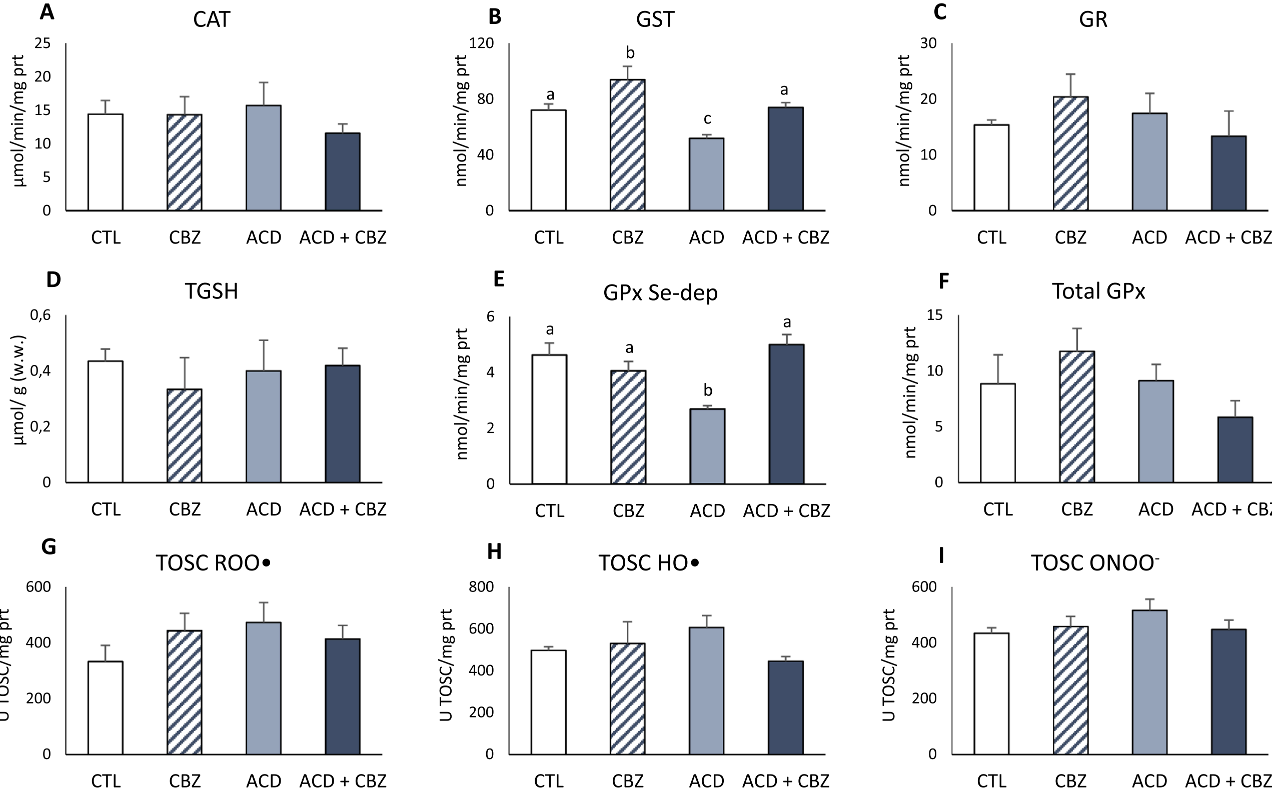

Fig. 4. Antioxidant defenses biomarkers in digestive glands of mussels exposed to various treatments. CAT: catalase (A), GST: glutathione S-transferase (B), GR: glutathione reductase (C), TGSH: total glutathione (D), Se-Dep. GPx: Se-dependent glutathione peroxidases (E) total GPx: sum of Se-dependent and Se-independent glutathione peroxidases (F), TOSC ROO•: total oxyradical scavenging capacity toward peroxyl radical (g), TOSC HO•: total oxyradical scavenging capacity toward hydroxyl radical (H), TOSC ONOO–: total oxyradical scavenging capacity toward peroxynitrite. Data are given as mean values ± Standard Error of the Mean (n = 5). Different letters indicate significant differences between group of means, CTL Control; CBZ Carbamazepine; ACD acidification; ACD + CBZ acidification + CBZ.

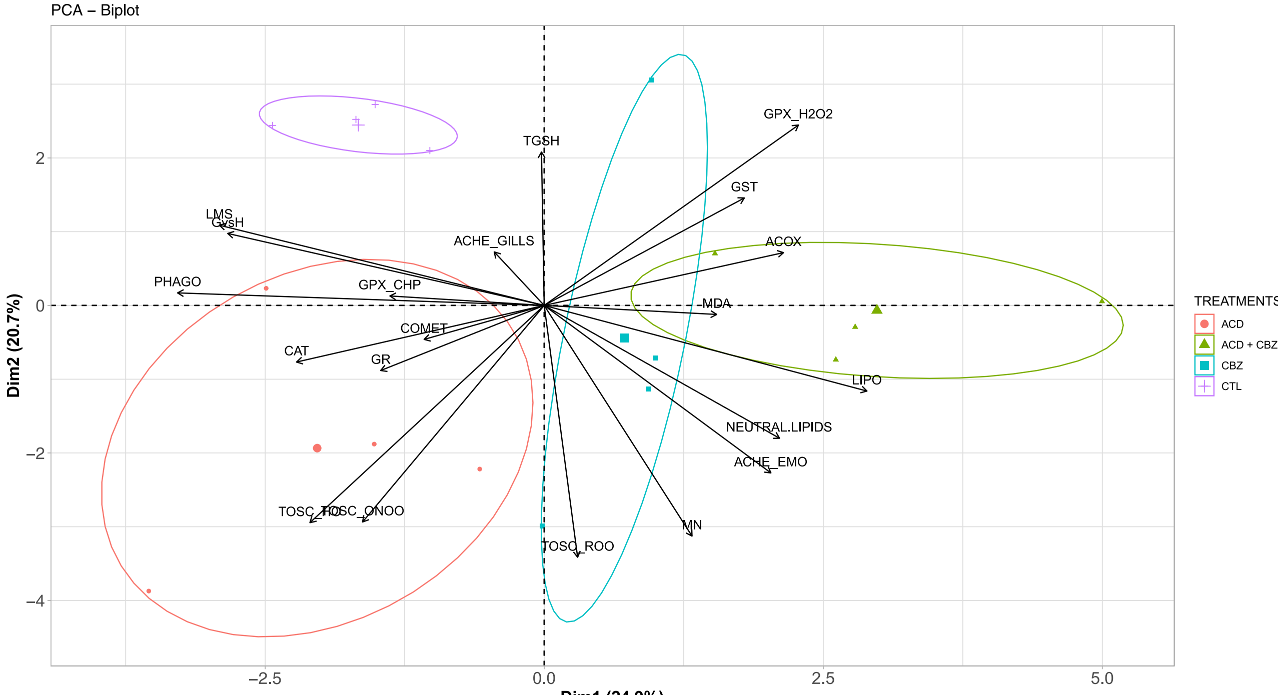

Fig. 5. Graphical representation of principal component analysis conducted on biological parameters analyzed in mussels tissues. CTL = Control; CBZ = Carbamazepine (1 µg/L) exposed; ACD = acidification (pH = 7.6); ACD + CBZ = acidification (pH = 7.6) + Carbamazepine (1 µg/L) d. Arrows represent all variables contribute to the separation.

Treatment	HQ	Class of Hazard	Level
CBZ	8.2	SLIGHT	
ACD	5.9	SLIGHT	
ACD + CBZ	14	MODERATE	

Fig. 6. Weight of Evidence (WOE) classification of biomarkers data for the whole dataset of analyzed parameters for each different laboratory condition. The quantitative hazard quotients (HQ) and the assigned class of hazard are given.

direct effects of carbamazepine in mussels synaptic transmission. In addition, putative *4-aminobutyrate aminotransferase* (GABT) belonging to "GABAergic synapse" KEGG pathway was 10 times down-regulated by CBZ (SM2). Possible tissue damage and inflammation were confirmed by the enriched terms "response to wounding" (10 DEGs), "wound healing" (8 DEGs), "cell death" (28 DEGs), "programmed cell death" (21 DEGs), "inflammatory response" (6 up-regulated genes) and "prostaglandin metabolic process" (3 up-regulated genes).

ACD exposed group revealed significant changes in genes involved in response to stress (41 DEGs), cell death (27 DEGs), apoptotic process (24 DEGs), and cellular response to DNA damage stimulus (6 DEGs) (Table 1 and SM2). A total of 6 transcripts coding for putative *heat shock protein 70* (*HSP70*), *heat shock protein 12A* (*HSP12A*) and *heat shock protein 12B* (*HSP12B*), as well as several genes involved in immune response were found up-regulated in response to acidification (Table 1 and SM2). Among the enriched GO terms identified by the functional analysis, it should be highlighted the BP_GO terms "cation transport" (16 DEGs), "ion homeostasis" (14 DEGs), the MF_GO terms "ion binding" (83 DEgs), "cation transmembrane transporter activity" (13 DEGs), "calmodulin binding" (7 DEgs) and the KEGG pathway "Calcium signaling pathway" (3 over-expressed genes). While functional analyses of down-regulated genes in ACD exposed mussels revealed the enriched GO_BP term "ion transport" represented by 8 DEGs, the vast majority of genes involved in ion homeostasis were up-regulated by ACD, as demonstrated by the enriched GO terms, "Calcium ion homeostasis" (9 up-regulated genes), "cellular ion homeostasis" (11 up-regulated genes), "inorganic ion homeostasis" (12 up-regulated genes) and "metal ion binding" (67 up-regulated genes) (Table 1). Among the significantly enriched terms, several biological processes were related to energy metabolism including "ATP metabolic process" (9 up-regulated genes), "energy derivation by oxidation of organic compounds" (12 up-regulated genes), "generation of precursor metabolites and energy" (12 up-regulated genes), and "energy reserve metabolic process" (6 up-regulated genes) (Table 1 and SM3).

Half of DEGs detected in mussels exposed to the combination of carbamazepine and acidification were differentially expressed only in response to this co-exposure (344 DEGs; 52%), while a total of 175 and 253 DEGs were common to those detected in response to the individual exposure to CBZ and ACD, respectively (Fig. 8). Among the genes differentially expressed only in response to ACD + CBZ, a total of 10 genes are involved in "calcium ion binding" and *Voltage-gated potassium channel subunit beta-3* (*KCAB3*). The ACD + CBZ exposures revealed significant changes also in transcriptional regulation of *Multidrug resistance-associated proteins* (*MRP5) and Multidrug resistance protein 1 (MDR1*) and two DEGs coding for *neuropeptide Y* (NYP). Additional up-regulated genes of immune response such as *Lysozyme 3* (*LYS3*) and *Complement C1q-like protein 4* (*C1QL4*) were also found differentially expressed only in ACD + CBZ exposed mussels. CBZ and ACD down-regulated *Heavy metal-binding protein* (HIP), while DNA damage and changes in apoptosis regulation are suggested by several genes involved in the cellular response to DNA damage stimulus, and by the up-regulation of *Caspase 2* and *Caspase 3, Protein shisa-5* (*SHISA5*), inhibitor of apoptosis proteins (*BIRC3* and *BIRC7*) and *DNA damage-regulated autophagy modulator protein 2* (*DRAM2*). Possible tissue damage and changes in apoptosis regulation and energy metabolism were supported by the enriched terms "Response to wounding" (9 DEGs), "Intrinsic apoptotic signaling pathway in response to DNA damage" (4 up-

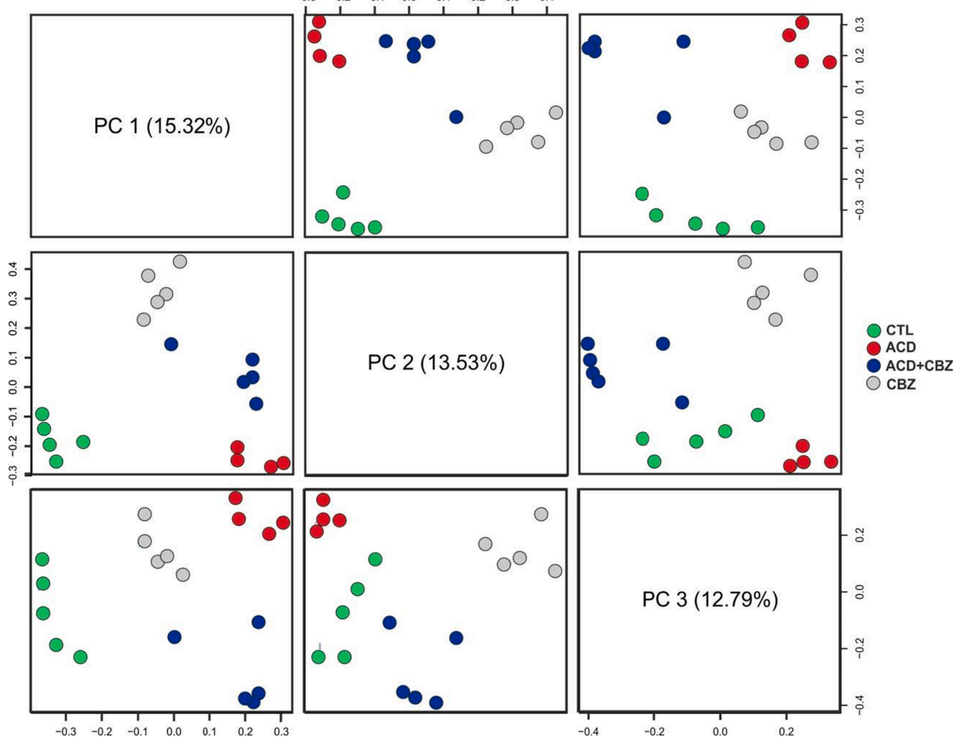

Fig. 7. Principal component analyses (PCA) of the entire set of genes considered for gene expression analyses.

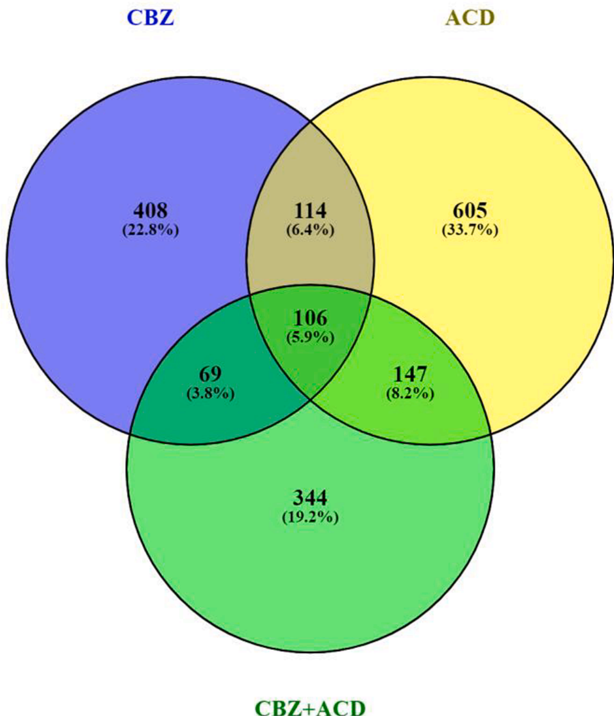

Fig. 8. Venn diagrams reporting for each pairwise comparison (CBZvsCTL; ACDvsCTL; ACD + CBZvsCTL) the number and percentage of DEGs.

regulated genes), "Energy derivation by oxidation of organic compounds" (7 DEGs) and "ATP generation from ADP" (3 up-regulated genes). Enrichment analyses identified also several biological processes and molecular pathways in common with the individual exposure to CBZ and ACD, such as ions transport, synapse, inflammation and immune response. The enriched biological processes "Reproduction" (22 DEGs) and "Sodium ion transport" (5DEGs), including putative *Transient receptor potential cation channel subfamily M member 2* (*TRPM2*), were found enriched only in ACD + CBZ (Table 1; SM3).

4. Discussion

4.1. Carbamazepine bioaccumulation and excretion under ocean acidification scenario

The need to understand the effects of multiple stressors is a major research issue and clear interactions between carbamazepine exposure and future projection of ocean acidification were provided by the present investigation. Uptake, bioaccumulation and excretion are key processes for risk assessment of pharmaceuticals to non-target species but limited pharmacokinetics and pharmacodynamics studies are actually available. CBZ is one of the most persistent pharmaceuticals in aquatic environment (Almeida et al., 2014, 2015, 2017; Miller et al., 2019), as confirmed by concentrations measured in control mussels tissues (37.7 ng/g d.w) which are well within the range of baseline values measured in mussels from unpolluted areas of the Adriatic Sea (Mezzelani et al., 2020).

Our study also revealed a significant uptake of CBZ in mussels exposed to environmentally realistic levels of this drug either when dosed alone or in combination with lower pH, without any influence of the latter on bioaccumulation. The limited time-dependent variations in carbamazepine accumulation during CBZ-exposure phase, suggests a rapid steady-state condition, as reported in previous studies with this drug (Boillot et al., 2015; Serra-Compte et al. 2018) and other pharmaceuticals such as the Non-steroidal Anti-Inflammatory Drugs (NSAIDs) Diclofenac and Nimesulide (Mezzelani et al., 2018a). Mussels do not typically possess the capability to regulate intracellular concentration of traditional chemical pollutants, but a certain metabolism of pharmaceuticals has been hypothesized (Boillot et al., 2015; Bonnefille et al., 2017; Serra-Compte et al. 2018; Swiacka et al., 2019). The principal metabolic pathway of CBZ in mammals is mediated by the cytochrome *P*-450 monooxygenases leading to formation of the carbamazepine-10,11-epoxide, followed by hydration to trans-10,11-dihydroxy-10,11-dihydro-carbamazepine and conjugation with glucuronic acid. The activity of Cytochrome P450 enzymes is generally very low in invertebrates, and the present study revealed a moderate effect of CBZ on biotransformation enzymes such as glutathione S-transferases. In addition, gene expression profile of CBZ-exposed mussels highlighted the up-regulation of genes involved in drug metabolism, i.e. *CYP3A2*, *CYP3A29*, *CYP4F8* and *ST1B1*, suggesting the induction of a rudimental CBZ metabolism in marine mussels. Nevertheless, the recalcitrant behavior of this drug to be fully metabolized and excreted was supported by the limited decrease of CBZ in exposed mussels after 10 days of depuration. Further, detectable levels of CBZ were still present in control mussels after 10 days of acclimation and additional 14 days of experiment, confirming that more than 20 days of depuration are needed to eliminate CBZ levels from mussels tissues (Serra-Compte et al., 2018).

Although ocean acidification can influence both the speciation and bioavailability of several environmental pollutants (Götze et al., 2014; Nardi et al., 2017), our study did not reveal significant differences of CBZ uptake in mussels exposed at normal or reduced pH, as also reported for *Scrobicularia plana* and *Ruditapes philippinarum* (Almeida et al. 2018; Freitas et al. 2016; Serra-Compte et al. 2018;). The high pKa of CBZ (13.9) would prevent a marked ionizable tendency at tested environmentally realistic pH projections, explaining the similar bioavailability; this could allow to hypothesize that ocean acidification would not represent a factor affecting CBZ bioavailability in future oceans, although interaction between these factors was evident in terms of biological effects.

4.2. Immune system alterations

Similar to contaminants mixtures, multiple environmental stressors can elicit synergistic, additive or antagonistic effects, depending on stressors combinations, investigated endpoint and level of biological organization (Heye et al., 2019). One of the main outcomes of this work revealed hemocytes as a target of CBZ, pH and of their interactions. Circulating hemocytes of mussels are involved in important functions, such as cell physiology, intracellular turnover, immune defense, wound healing, metabolite transport, shell deposition, degradation and elimination of pathogens (Gorbi et al., 2013; Munari et al., 2019). In the present study, a significant reduction of lysosomal membrane stability was observed in mussels exposed to CBZ, both at control pH and hypercapnic condition, with the lowest values in organisms co-exposed to both stressors. A similar effect was observed on the inhibition of phagocytosis capacity, which represents one of the most important cell-mediate immune responses in mussels, enabling specific hemocytes subpopulations to recognize and eliminate non-self-components. In *M. galloprovincialis* granulocytes are responsible of phagocytosis, and the observed inhibition of this activity can thus be explained by a decrement of circulating granulocytes, as supported by the lowered granulocytes vs hyalinocytes ratio due to CBZ-exposure. Although reduced LMS due to CBZ exposure was also reported in *R. philippinarum* (Aguirre-Martínez et al., 2016), the combined modulation of mussels immune responsiveness toward such multiple stressors, was so far never highlighted. In addition, the significant induction of acetylcholinesterase activity in all exposed mussels would support the proposed role of this enzymatic activity in modulation of the immune response of bivalves hemocytes during disturbance (Gerdol et al., 2018; Shi et al., 2014). Worthy to note, the weaker effect of CBZ on AChE induction under hypercapnic condition compared to control pH, allows to hypothesize antagonistic

Table 1
Lists of differentially expressed genes involved in different biological processes and molecular pathways. Enriched GO terms and pathway are reported in bold (↑ and ↓ indicated enriched terms considering up- and down-regulated genes separately). Number of differentially expressed genes for each terms are also reported, as well as the gene name of differentially expressed genes (green: down-regulated genes in exposed mussels; red: up.regulated genes in exposed mussels). Full list of DEGs are reported in Supplementary Material 2 SM2 including the gene description for each Gene ID. Full lists of enriched terms are reported in Supplementary Material SM3.

PATHWAY / BIOLOGICAL PROCESSES	N° DEGs	GENE NAME
ACD vs CTL		
Response to stress	41	CNOT1; KSYK; RAD54; PDE1; FA8; FAK1; PPIP1; M4K4; CO3A1; VIT6; CAP1; PLMN; MRC1; COIA1; MMGL; FCN2; NPL4; DCAM; IF4A; OAS1; SRSF6; STML2; SRSF6; DTX3L; COL12; CYAD; TRI25; JAK2; BPI; TFPI2; LIG1; LRC33; HRH4; ZN622; MYSN; C1QT3; CXXC1; XIAP; DMBT1; CREBRF; FMAR
Cell death	27	BIR7B; MBL; FAK1; M4K4; COIA1; IP3KB; PDE1A; T22D1; PDE1B; SRSF6; MEF2A; SRSF6; DNAJB6 CASP3; JAK2; TIF1A; MEG10; GRAM4; DRAM2; LITFL; ANK2; PEN2; CASP3; ZN622; PIM3; XIAP; FUTSC
Apoptotic process	24	BIRC7B; MBL; FAK1; M4K4; COIA1; IP3KB; PDE1A; T22D1; PDE1B; SRSF6; MEF2A; DNAJB6; CASP3; JAK2; MEG10; GRAM4; DRAM2; LITFL; PEN2; CASP3; ZN622; PIM3; XIAP; FUTSC
Cellular response to DNA damage stimulus	6	CNOT1; LIG1; IF4A; RAD54; XIAP; DTX3L
Immune response	22	KSYK; FAK1; PPIP1; CO3A1; PLCG1; MRC1; FCN2; OAS1; STML2; COL12; CL004; TRI25; JAK2; BPI; LRC33; DMBT1; MYTB; MYNA; BPI; C1QL3; PLA2GE
Ion binding	83	ARFG2; FBCD1; ZN474; ZN891; BIR7B; P4HA2; MBL; MBL; MAB3; PDE1; FA8; CALU; TSSK2; ZC3H1; SC5D; SLIT1; CSTN1; ATS2; PYGM; CO3A1; PLCG1; ATC1; ITA8; COIA1; COX1; ARSB; MATN1; PDE1A; NCAN; FCN2; NPL4; SPAN; PGBM; CYB; PDE1B; DCHS; MIG17; UNC22; RO60; CAZ; DTX3L; COL12; LIMA; FNKE; CYAD; CYGB1; T23O; TRI25; JAK2; DYH7; TIF1A; PC11X; PPIG; ZN544; ODO1; RNF44; ZSCA2; PPN; LIG1; EFHA2; I17RD; FRRS1; VWDE; HMCN2; ZCH10; TITIN; DYST; CLIP1; FBCD1; T23O; ZN622; KCTD7; HMCN1; CAD23; TSSK4; DZAN1; DSPP; MARK1; CXXC1; ZNFX1; XIAP; NR2E3; IDH3A
Ion homeostasis	13	FAK1; PLCG1; ATC1; ANK2; TRPM1; STML2; JAK2; TIF1A; HRH4; CAD23; COPT2; COPT1; FMAR
Ion transport ↓	8	S27A2; ANK2; S22AD; SC6A7; ASI1A; CAD23; AMT2; COPT2
Metal ion binding / Inorganic ion homeostasis/ Cellular ion homeostasis ↑	74	ARFG2; FBCD1; ZN474; ZN891; BIR7B; P4HA2; MBL; MAB3; PDE1; FA8; FAK1; CALU; TSSK2; ZC3H1; SC5D; SLIT1; CSTN1; ATS2; PLCG1; ATC1; ITA8; COIA1; COX1; ARSB; PDE1A; NCAN; FCN2; NPL4; PGBM; CYB; PDE1B; OAS1; MIG17; CAZ; TRPM1; STML2; LIMA; FNKE; CYAD; CYGB1; T23O; JAK2; DYH7; TIF1A; PC11X; PPIG; ZN544; ODO1; RNF44; ZSCA2; PPN; LIG1; EFHA2; ANK2; FRRS1; VWDE; HMCN2; DYST; HRH4; CLIP1; FBCD1; T23O; KCTD7; COPT2; TSSK4; COPT1; DZAN1; DSPP; MARK1; CXXC1; ZNFX1; XIAP; IDH3A; FMAR
Calcium ion homeostasis ↑	9	PLCG1; ATC1; TRPM1; STML2; JAK2; TIF1A; ANK2; HRH4; FMAR;
Calmodulin binding*	7	PDE1; IP3KB; PDE1A; PDE1B; PHKG1; UNC22; TITIN;
Calcium signaling pathway ↑	3	PDE1A; PDE1B; PHKG1
Generation of precursor metabolites and energy ↑	12	PYGM; INSR; COX1; CYB; NU4M; NU5M; PHKG1; PPIG; ODO1; AAKG2; SRBS1; IDH3A
ATP metabolic process↑	9	INSR; COX1; ATP6; CYB; NU4M; NU5M; STML2; ODO1; AAKG2
CBZ vs CTL		
Cell Cycle	18	BIRC7; CSPP1; CHM2A; LIG1; CHFR; EPS8; HMCN1; MIF; SIR1; LARK; SEPT7; SRSF2; SC6A4; SPG20; ERCC2; TITIN; VPS4B
Immune Response	9	MYNA; LYZ1; C1QL3, DEFI, MRC; IRG1, IF27B, LITAF, PLA2
DNA repair	7	LIG1; RAD54; MMS22; SIR1; ERCC2; KAT5; HERC2
Drug metabolism/oxidative stress	4	SESN3; CYP4F8; ST1B1; CYP3A2, CYP3A29; CYP2E1
Apoptotic process	23	BIRC1; BIRC2; BIRC7; CHIA; DNAJB6; ERCC2; FAK1; FGD1; G2E3; GIMAP4; GIMAP7; HIP1; IP3KB; LITFL; MAX; MBL; MEG10; MIF; ORCT; RBM25; RHBD1; SFRP5; SIR1
Cell death	28	ANK2; BIRC1; BIRC2; BIRC7; CHIA; DNAJB6; ERCC2; FAK1; FGD1; G2E3; HIP1; IP3KB; LITFL; MAX; MBL; MEG10; MIF; MMP3; ORCT; RBM25; RBM25; RHBD1; SFRP5; SIR1; TCPA; TIF1A; VPS4B
Synapses	17	SC6A4; CE112; SPG20; SYNG1; FUS; SYTL4; ESPN; BAI3; ANK2; HIP1; DYST; STX7; EPS8; HRH4; SEPT7; PDZD2; DLG1
Regulation of synapse organization	6	SYNG1; BAI3; C1QL3; CAZ; FAK1; MDGA1
Receptor localization to synapse↑	3	CE112; STX7; DLG1
Response to wounding	10	ILK; MAX; GABT; MMGL; BIRC1; MYL9; FA8; TFPI2; XCT; IFRD1
Inflammatory response↑	6	MMGL; BIRC1; FA8; IRG1; C163A; MIF; HRH4
ACD+CBZ vs CTL		
Apoptotic process	19	E41L3; ARHGC; NHRF1; BIRC3; ATRX; ERCC2; SRPK2; MIF; SHSA5; LRP6; TIAR; CASP2; TRI39; CASP3; T22D1; DRAM2; BIR7A; HNRPK; SRSF6
Cellular response to DNA damage stimulus	9	DOT1L; CENPX; SETD2; ATRX; ERCC2; MIF; SHSA5; CASP2; HNRPK
Immune system process↑	18	EPS8; CS066; LYAM3; ERCC2; SRPK2; MIF; OAS1; PLMN; PLCG1; DCTN4; GSLG1; TRPM2; TITIN; FCN2; CASP3; MDR1; MRC1; PLMN
Ion transport	18	GABT; S22AD; AMT2; S27A2; SO5A1; SC5A8; S39AE; AT1B1; MRP5; NHRF1; CNG1; MIF; S17A5; PLCG1; TRPM2; S23A1; ANK2; UNC9
Calcium ion binding	19	ANXA7; HMCN1; TITIN; TRHY; RFP4A; LRP4; PC11X; GLU2B; LRP; PLCG1; PCDH7; SLIT1; NCS2; MMP3; TRPM2; HMCN2; DSPP
Regulation of cytosolic calcium ion concentration↑	5	LRP6; PLCG1; ANK2; TRPM2; FMAR
Calcium-mediated signaling↑	4	PLCG1; LYAM3; ANK2; TRPM2
Sodium ion transport	5	NHRF1; SC5A8; TRPM2; AT1B1; S23A1
Synapse organization↑	6	LRP4; BAI3; SLIT1; MDGA1; SPTCB; KY
Response to wounding	9	GABT; MMGL; LYAM3; NINJ2; PLMN; IFRD1; TFPI2; SRSF6; PLMN
Intrinsic apoptotic signaling pathway in response to DNA damage↑	4	MIF; SHSA5; CASP2; HNRPK
Energy derivation by oxidation of organic compounds	7	INSR; GLYG; IDH3A; GLYG2; PYGM; PPIG; ODO1
ATP generation from ADP↑	3	ODO1; INSR; AKD1
Reproduction	25	UNC22; VIT6; VEZP9; VEZP12, VERL, GABT CENPX; CHDM; AT1B1; SETD2; DOT1L; INSR; LRP6; TIAR; PLMN; WBP2L; SPTCB; SP17; ATRX; TBD; CCD63; CO4A1; CYAD; CBR1; CASP2

interactions between tested stressors.

In analogy with cellular biomarkers, gene expression analyses in digestive glands highlighted the significant up-regulation of several genes with a key role in immune response and inflammation (e.g. *MYNA*, *LYZ1*, *DEFI*, *MRC1*, *IRG1*, *LITAF* and *PLA2* among others), suggesting a coordinated activation of mussels immune defenses in response to CBZ exposure. Although immune system has not been characterized as direct target of CBZ in mammals, an increasing body of evidence indicates immunosuppression as side effect due to alteration of membrane phospholipids, inhibition of protein synthesis in lymphocytes, modulation of plasma levels of different interleukins, and a significant increment in cytotoxic activity of NK cells (Beghi and Shorvon, 2011). Results obtained in the present study corroborate such evidence also for non-target marine invertebrates and highlight an exacerbation of these effects under ocean acidification condition. A large number of genes involved in immunological pathways, appeared to be affected by hypercapnia: the significant up-regulation of *MRC1, FCN2, MYTB, MYNA* and C1QL3 in ACD-exposed mussels, highlighted the earlier responsiveness of transcriptional level compared to cellular biomarkers. The combination of CBZ and low pH caused the most important activation of immune response among treatments, as shown by the enriched term “Immune system process” represented by 18 up-regulated genes (Table 1), and thus confirming that interactive effects on immune system progressed from molecular up to cellular level.

Among the DEGs only in response to combination of stressors, a significant up-regulation was observed for *Lysozyme 3* (*LYS3*) and *Complement C1q-like protein 4* (*C1QL4*), which act as a critical first-line responder of innate immunity (Leprêtre et al., 2019). An increased number of studies on bivalve species demonstrated that environmental stressors may have a direct effect on microbial composition or/and lead to the decreased host ability to control the symbiotic microbial communities (Milan et al., 2018b; Milan et al., 2019). Overall, from our findings, biochemical and molecular modifications in hemocytes and immune response could partially reflect the attempt to control changes in host-microbiota compositions, suggesting that future studies are needed to understand possible links between host-immune system activation and possible changes related to CBZ and hypercapnia exposures.

4.3. Carbamazepine mechanism of action in M. galloprovincialis

Carbamazepine has three main MoA in mammals species: *i)* it inhibits the depolarization of neurons through the enhancement of inhibitory neurotransmission mediated by the gamma-aminobutyric acid (GABA); *ii)* it increases the concentration of the inhibitory neurotransmitter serotonin at neuronal synapses; *iii)* it binds to the voltage-gated sodium ion channels proteins, blocks the channel and reduces the frequency at which impulses are fired during epileptic crisis (Ambrosio et al., 2002; Harkin & Hopkinson, 2010; Ofoegbu et al., 2016).

Analyses on transcriptional profiles provided insights on possible similarities between CBZ MoA in invertebrates and vertebrates, including the activation of a large number of molecular mechanisms related to synapses and neurotransmission in CBZ-exposed mussels. First, the direct involvement of gamma-aminobutyric acid (GABA) pathway is suggested by the down-regulation in CBZ-exposed mussels of *GABA transaminase* (*GABT*), responsible for GABA catabolism. Second, the significant modulation of *Sodium-dependent serotonin transporter (SC6A4),* representing an integral membrane protein that transports the neurotransmitter serotonin from synaptic spaces into presynaptic neurons, suggests additional MoA similarities among species. In particular, the *SC6A4* down-regulation in CBZ exposed mussels may lead to a significant increase of extracellular serotonin concentration, which represents a typical effect of CBZ exposure in mammals (Dailey et al., 1998). Third, the combined effects of CBZ and hypercapnia led to significant changes in genes involved in “Sodium ion transport” that can be related to the blockade of inactivated neuronal sodium channels, one of the main CBZ MoA in vertebrate species (Suwalsky et al. 2006). Among genes involved in this pathway particular attention should be payed to the up-regulation of *TRPM2* coding for a protein forming a tetrameric cation channel permeable to calcium, sodium, and potassium. *TRPM2*, already described to be up-regulated in bivalves in response to chemicals exposures (Ertl et al., 2016), is activated by oxidative stress and mediates mitochondria-dependent apoptosis by increasing calcium influx (Kang et al., 2018; Nazıroğlu and Demirdaş, 2015; Takahashi et al. 2011).

Also the induction of acetylcholinesterase activity observed in CBZ-exposed mussels reveals a certain parallelism between carbamazepine MoA in vertebrate and invertebrates species. Indeed, CBZ is a mood stabilizer, used in human medicine to treat epilepsy, neuropathic pain and maniac disorders. As mentioned above, voltage-gated sodium ion channels represent the main target of CBZ, but it has also been demonstrated its capability to interact with voltage-gated calcium and potassium ion channels, cholinergic, purinergic systems, signaling pathways (serotonergic, dopaminergic, glutamatergic) and receptors (Ambrosio et al., 2002, Siebel et al., 2010). In this respect, the AChE is necessary for the hydrolysis of the neurotransmitter acetylcholine in cholinergic synapses and plays a critical role in the nervous system. Mizuno et al. (2000), observed that therapeutic doses of CBZ enhanced both acetylcholine release and synthesis in rat striatal and hippocampal brain tissues, demonstrating the modulation of cholinergic system. In non-target species a certain responsiveness of this pathway was observed, but without a unique pattern of variation: it was inhibited in *R. philippinarum* treated with 1 μg/L of CBZ, while induced in the amphipod *Ampelisca brevicornis* exposed to sediments spiked with carbamazepine at 0.5 ng/g (Aguirre-Martínez et al., 2016; Maranho et al., 2015). Overall, results obtained in this study confirmed that some pathways of human pharmaceuticals are preserved among species, being modulated also in non-target organisms (Almeida et al., 2018; Aguirre-Martínez et al., 2016; Ofoegbu et al., 2016).

4.4. Oxidative and genotoxic effects

Contrasting results were observed on typical markers of genotoxicity measured in terms of DNA strand breaks or MN frequency. The lack of DNA damage in hemocytes through the Comet assay could be explained by efficient DNA repair mechanisms, as also supported by modulation of genes belonging to these pathways in mussels from all experimental treatments. On the other hand micronuclei frequency increased under reduced pH conditions, allowing to hypothesize that hypercapnia can affect hemocytes cell cycle, as already highlighted in *M. galloprovincialis* and in the scallop *Felxopecten glaber* (Nardi et al., 2017, 2018a, 2018b): the observed micronuclei increment, marker of damage at chromosome level, is supported by the significant upregulation of genes involved in apoptosis in digestive gland of organisms exposed to hypercapnia.

One of the main mechanisms causing the onset of DNA damage is mediated through increased intracellular production of reactive oxygen species (ROS) or impaired efficiency of the antioxidant system (Regoli and Giuliani, 2014). Our study, through the integrated measurement of individual antioxidants and the capability to neutralize specific oxyradicals, evidenced a rather weak involvement of this pathway: limited differences of catalase, total glutathione and glutathione-dependent enzymes, were paralleled by the lack of consistent variations of the total oxyradical scavenging capacity toward peroxynitrite, hydroxyl and peroxyl radicals. These findings indicate that prooxidant mechanisms do not represent the primary mode of action of these environmental stressors.

However, histochemical analyses revealed cumulative and interactive effects of CBZ and hypercapnic condition, on respectively lipofuscin and neutral lipids content. Accumulation of lipofuscin, a typical end product of membrane peroxidation is generally accompanied by lysosomal alterations due to its capability to bind lysosomal hydrolases, with

consequent inhibition of protein degradation and autophagic accumulation of undegradable damaged organelles, proteins, phospholipids and lipids, contributing to further lipofuscin formation (Moore et al., 2006). The accumulation of neutral lipids, that represent one of the major energy reserves in bivalves, is considered an indicator of disturbed metabolism (Bocchetti and Regoli 2006). An increment of lipofuscin under ocean acidification scenario was observed in *M. galloprovincialis*, *Euplotes crassus* and *Sterechinus neumayeri*, while the simultaneous increase of lipofuscin and neutral lipids was reported in *M. galloprovincialis* treated with CBZ (Dell'Acqua et al., 2019; Gomiero et al., 2018; Martin-Diaz et al., 2009; Nardi et al., 2017).

Acidification conditions can alter the intracellular milieu (Ivanina and Sokolova, 2015; Stillman and Paganini, 2015), and our RNAseq results on ACD and ACD + CBZ exposed mussels confirmed changes in gene expression of ion binding, transport and homeostasis processes. The subsequent impairment of cellular homeostasis could be the cause of a complex cascade of events involving gene alterations of calcium signaling pathway, mitochondrial energy metabolism (COX, ATP6, IDH3A, ODO1 among others) and the up-regulation of apoptotic pathways (CASP2, CASP3 among others). This cascade, when combined with CBZ-exposure, could explain the peroxidation processes observed herein in terms of accumulation of lipofuscin and neutral lipids in lysosomes, along with the induction of proteins repair mechanisms (*HSP70*). In this context, previous studies highlighted the involvement of calcium-signaling pathway in the accumulation of lipofuscin in human retinal pigment epithelial cells (Zhang et al., 2011) and in both lysosomal alterations and oxidative insult in *M. galloprovincialis* (Dailianis et al., 2009; Raftopoulou et al., 2006).

4.5. Additional interactive effects of carbamazepine and hypercapnia

Several changes detected in response to ACD + CBZ, such as apoptosis, inflammation, immune response, ions transport, synapse and DNA damage showed enhanced but similar trends to those revealed in the individual exposures, suggesting cumulative effects of combined stressors. On the other hand, the 52% of DEGs detected in mussels exposed to the combination of carbamazepine and acidification were differentially expressed only in response to this treatment. Among them, genes involved in "calcium ion binding" "Sodium ion transport" and "Potassium channel" were found differentially expressed. While the capability of CBZ to interact with voltage-gated sodium ion channels and voltage-gated calcium and potassium ion channels have already been discussed above, the effects of OA in the homeostasis of carbonate and calcium ions have been reported in several studies (Beniash et al. 2010; Richards et al. 2018), suggesting possible synergistic effects of the two stressors on ion homeostasis. Nonetheless, the multidrug resistance proteins *MRP5*, having a role in cell detoxification through their ability to excrete xenobiotic conjugates and metabolites, were down-regulated in organisms co-exposed to CBZ and reduced pH, potentially explaining the slightly delayed elimination of CBZ in organisms exposed to this molecule under reduced pH scenario. In addition, the biological process "Reproduction" was enriched only in mussels exposed to ACD + CBZ, and six down-regulated genes coding for vitellogenin like transcripts (*Vitellogenin 6,* VIT6; *vitelline envelope zona pellucida domain protein 12, VEZP12*; *vitelline envelope zona pellucida domain protein 9, VEZP9*; *putative vitelline envelop receptor for lysin, VERL*) were also found. Although the controversial role of vitellogenin in marine mussels, to our knowledge, just few studies identified potential effects of carbamazepine and ocean acidification on vitellogenin like transcripts (Harms et al., 2014).

Overall, gene expression profiling indicated several pathways where combined stressors induced additive or even synergistic effects, thus representing a potential risk for bivalve populations exposed to pharmaceuticals under future scenarios of reduced pH.

Single and combined effects of CBZ and acidification in *M. galloprovincialis* were further supported when all the cellular responses were elaborated through the weighted criteria of the quantitative Sediqualsoft model, which summarizes a hazard index based on the number of changed biomarkers, the toxicological relevance of measured end points and magnitude of variations compared to threshold specific for each response (Benedetti et al., 2012; Piva et al., 2011). With this approach, the calculated HQ for biomarkers raised from "Slight" in mussels exposed to individual stressors, to "Moderate" in organisms co-exposed to CBZ and hypercapnia.

5. Conclusions

This study provided clear evidences of interactive effects of CBZ and acidification in *M. galloprovincialis*. Despite hypercapnia had a limited influence on CBZ accumulation, modulation of ecotoxicological effects was highlighted by changes in gene expression profile and functional biological responses at cellular level. Transcriptional responses showed the early activation of immune response, inflammation processes, apoptosis, DNA damage, ions transport and changes in energy metabolism. Such alterations progressed from molecular up to functional cellular level, with a frequent synergistic, additive and rather antagonistic, action of CBZ and reduced pH. The integration of chemical, molecular and cellular findings allowed to hypothesize intricate pathways behind the biological effects of acidification and carbamazepine, suggesting potential threats for the health status of *M. galloprovincialis* in coastal areas characterized by noticeable carbamazepine levels. Given the importance of this species in ecosystem functioning and its economical relevance in seafood market additional long-term studies are recommended to unravel the ecological consequences of contaminants of emerging concern under predicted scenarios of ocean changes.

CRediT authorship contribution statement

Marica Mezzelani: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Alessandro Nardi:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Ilaria Bernardini:** Investigation, Formal analysis, Data curation. **Massimo Milan:** Investigation, Formal analysis, Data curation, Resources, Writing - review & editing. **Luca Peruzza:** Investigation, Formal analysis, Data curation. **Giuseppe d'Errico:** Software, Formal analysis, Data curation. **Daniele Fattorini:** Data curation, Supervision. **Stefania Gorbi:** Resources, Supervision. **Tomaso Patarnello:** Resources, Funding acquisition. **Francesco Regoli:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106269.

References

Aguirre-Martínez, G.V., DelValls, T.A., Martín-Díaz, M.L., 2016. General stress, detoxification pathways, neurotoxicity and genotoxicity evaluated in *Ruditapes philippinarum* exposed to human pharmaceuticals. Ecotox. Environ. Saf. 124, 18–31. https://doi.org/10.1016/j.ecoenv.2015.09.031.

Almeida, Â., Calisto, V., Esteves, V., Soares, A.M.V.M., Figueira, E., Freitas, R., 2014. Presence of carbamazepine in coastal systems: effects on bivalves. Aquat. Toxicol. 156, 74–87. https://doi.org/10.1016/j.aquatox.2014.08.002.

Almeida, A., Freitas, R., Calisto, V., Esteves, V.I., Schneider, R.J., Soares, A.M.V.M., Figueira, E., 2015. Chronic toxicity of the antiepileptic carbamazepine on the clam *Ruditapes philippinarum*. Comp. Biochem. Physiol C 172–173, 26–35. https://doi.org/10.1016/j.cbpc.2015.04.004.

Almeida, A., Calisto, V., Esteves, V.I., Schneider, R.J., Soares, A.M.V.M., Figueira, E., Freitas, R., 2017. Toxicity associated to uptake and depuration of carbamazepine in the clam *Scrobicularia plana* under a chronic exposure. Sci. Total Environ. 580, 1129–1145. https://doi.org/10.1016/j.scitotenv.2016.12.069.

Almeida, A., Freitas, R., Calisto, V., Esteves, V.I., Schneider, R.J., Soares, A.M.V.M., Figueira, E., Campos, B., Barata, C., 2018. Effects of carbamazepine and cetirizine under an ocean acidification scenario on the biochemical and transcriptome responses of the clam *Ruditapes philippinarum*. Environ. Pollut. 235, 857–868. https://doi.org/10.1016/j.envpol.2017.12.121.

Álvarez-Muñoz, D., Rodríguez-Mozaz, S., Maulvault, A.L., Tediosi, A., Fernández-Tejedor, M., Van den Heuvel, F., Kotterman, M., Marques, A., Barceló, D., 2015. Occurrence of pharmaceuticals and endocrine disrupting compounds in macroalgaes, bivalves, and fish from coastal areas in Europe. Environ. Res. 143, 56–64. https://doi.org/10.1016/j.envres.2015.09.018.

Ambrosio, A.F., Soares-Da-Silva, P., Carvalho, C.M., Carvalho, A.P. 2002. Mechanisms of action of carbamazepine and its derivatives, oxcarbazepine, BIA 2-093, and BIA 2-024. Neurochem Res. 27, 121-130. DOI: 10.1023/a:1014814924965.

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. https://doi.org/10.1016/j.envpol.2014.12.021.

Beghi, E., Shorvon, S., 2011. Antiepileptic drugs and the immune system. Epilepsia 52, 40–44. https://doi.org/10.1111/j.1528-1167.2011.03035.x.

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. https://doi.org/10.1016/j.envint.2011.08.003.

Beniash, E., Ivanina, A., Lieb, N.S., Kurochkin, I., Sokolova, I.M., 2010. Elevated level of carbon dioxide affects metabolism and shell formation in oysters Crassostrea virginica. Mar. Ecol. Prog. Ser. 419, 95–108. https://doi.org/10.3354/meps08841.

Birch, G.F., Drage, D.S., Thompson, K., Eaglesham, G., Mueller, J.F., 2015. Emerging contaminants (pharmaceuticals, personal care products, a food additive and pesticides) in waters of Sydney estuary. Australia. Mar. Pollut. Bull. 97, 56–66. https://doi.org/10.1016/j.marpolbul.2015.06.038.

Bocchetti, R., Regoli, F., 2006. Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic Sea. Chemosphere 65, 913–921. https://doi.org/10.1016/j.chemosphere.2006.03.049.

Boillot, C., Martinez Bueno, M.J., Munaron, D., Le Dreau, M., Mathieu, O., David, A., Fenet, H., Casellas, C., Gomez, E., 2015. In vivo exposure of marine mussels to carbamazepine and 10-hydroxy-10,11-dihydro-carbamazepine: bioconcentration and metabolization. Sci. Total Environ. 532, 564–570. https://doi.org/10.1016/j.scitotenv.2015.05.067.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Bonnefille, B., Arpin-Pont, L., Gomez, E., Fenet, H., Courant, F., 2017. Metabolic profiling identification of metabolites formed in Mediterranean mussels (Mytilus galloprovincialis) after diclofenac exposure. Sci. Total Environ. 583, 257–268. https://doi.org/10.1016/j.scitotenv.2017.01.063.

Bray, N.L., Pimentel, H., Melsted, P., Pachter, L., 2016. Near-optimal probabilistic RNA-seq quantification. Nat. Biotechnol. 34, 525–527. https://doi.org/10.1038/nbt.3519.

Cao, R., Liu, Y., Wang, Q., Dong, Z., Yang, D., Liu, H., Ran, W., Qu, Y., Zhao, J., 2018. Seawater acidification aggravated cadmium toxicity in the oyster *Crassostrea gigas*: Metal bioaccumulation, subcellular distribution and multiple physiological responses. Sci. Total Environ. 642, 809–823. https://doi.org/10.1016/j.scitotenv.2018.06.126.

Cao, R., Zhang, T., Li, X., Zhao, Y., Wang, Q., Yang, D., Qu, Y., Liu, H., Dong, Z., Zhao, J., 2019. Seawater acidification increases copper toxicity: A multi-biomarker approach with a key marine invertebrate, the Pacific Oyster *Crassostrea gigas*. Aquat. Toxicol. 210, 167–178. https://doi.org/10.1016/j.aquatox.2019.03.002.

Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee. European Union Strategic Approach to Pharmaceuticals in the Environment COM/2019/128 final, 2019.

Dailey, J.W., Reith, M.E.A., Steidley, K.R., Milbrandt, J.C., Jobe, P.C., 1998. Carbamazepine-induced release of serotonin from rat hippocampus in vitro. Epilepsia 39, 1054–1063. https://doi.org/10.1111/j.1528-1157.1998.tb01290.x.

Dailianis, S., Patetsini, E., Kaloyianni, M., 2009. The role of signalling molecules on actin glutathionylation and protein carbonylation induced by cadmium in haemocytes of *mussel Mytilus galloprovincialis* (Lmk). J. Exp. Biol. 212, 3612–3620. https://doi.org/10.1242/jeb.030817.

Dell'Acqua, O., Ferrando, S., Chiantore, M., Asnaghi, V., 2019. The impact of ocean acidification on the gonads of three key Antarctic benthic macroinvertebrates. Aquat. Toxicol. 210, 19–29. https://doi.org/10.1016/j.aquatox.2019.02.012.

Delorenzo, M.E., 2015. Impacts of climate change on the ecotoxicology of chemical contaminants in estuarine organisms. Curr. Zool. 61, 641–652. https://doi.org/10.1093/czoolo/61.4.641.

Dennis, G., Sherman, B.T., Hosack, D.A., Yang, J., Gao, W., Lane, H., Lempicki, R.A., 2003. david: Database for annotation, visualization, and integrated discovery. Genome Biol. 4 (9), R60. PMID: 12734009.

Dickson, A.G., Sabine, C.L., Christian, J.R., 2007. Guide to best practices for ocean CO_2 measurements. PICES Special Publication 3, 191.

Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Text with EEA relevance. 2010.

Ertl, N.G., O'Connor, W.A., Brooks, P., Keats, M., Elizur, A., 2016. Combined exposure to pyrene and fluoranthene and their molecular effects on the Sydney rock oyster. *Saccostrea glomerata*. Aquat Toxicol. 177, 136–145. https://doi.org/10.1016/j.aquatox.2016.05.012.

Franzellitti, S., Balbi, T., Montagna, M., Fabbri, R., Valbonesi, P., Fabbri, E., Canesi, L., 2019. Chemosphere Phenotypical and molecular changes induced by carbamazepine and propranolol on larval stages of *Mytilus galloprovincialis*. Chemosphere 234, 962–970. https://doi.org/10.1016/j.chemosphere.2019.06.045.

Freitas, R., Almeida, A., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2016. The impacts of pharmaceutical drugs under ocean acidification: new data on single and combined long-term effects of carbamazepine on *Scrobicularia plana*. Sci. Total Environ. 541, 977–985. https://doi.org/10.1016/j.scitotenv.2015.09.138.

Gaw, S., Thomas, K.V., Hutchinson, T.H., 2014. Sources, impacts and trends of pharmaceuticals in the marine and coastal environment. Philos. Trans. R. Soc. Lond. B Biol. Sci. 369, 1656. https://doi.org/10.1098/rstb.2013.0572.

Gazeau, F., Parker, L.M., Comeau, S., Gattuso, J.-P., O'Connor, W.A., Martin, S., Pörtner, H.-O., Ross, P.M., 2013. Impacts of ocean acidification on marine shelled molluscs. Mar. Biol. 160 (8), 2207–2245. https://doi.org/10.1007/s00227-013-2219-3.

Gerdol M., Gomez-Chiarri, M., Castillo, M.G., Figueras, A., Fiorito, G., Moreira, R., Novoa, B., Alberto Pallavicini, A., Ponte, G., Roumbedakis, K., Venier, P., Vasta, G.R. Immunity in Molluscs: Recognition and Effector Mechanisms, with a Focus on Bivalvia. In: Cooper E. (eds) Advances in Comparative Immunology. Springer, Cham DOI: 10.1007/978-3-319-76768-0_11.

Giuliani, M.E., Filippini, G., Nardi, A., 2020. Season specific influence of projected ocean changes on the response to cadmium of stress-related genes in Mytilus galloprovincialis. Mar. Environ. Res. In press. https://doi.org/10.1016/j.marenvres.2020.105091.

Gomiero, A., Bellerby, R., Zeichen, M.M., Babbini, L., Viarengo, A., 2018. Biological responses of two marine organisms of ecological relevance to on-going ocean acidification and global warming. Environ. Pollut. 236, 60–70. https://doi.org/10.1016/j.envpol.2018.01.063.

Gorbi, S., Avio, G.C., 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 Immun. 35, 941–950. https://doi.org/10.1016/j.fsi.2013.07.003.

Götze, S., Matoo, O.B., Beniash, E., Saborowski, R., Sokolova, I.M., 2014. Interactive effects of CO2 and trace metals on the proteasome activity and cellular stress response of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. Aquat. Toxicol. 149, 65–82. https://doi.org/10.1016/j.aquatox.2014.01.027.

Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B., Eccles, D., Li, B., Lieber, M., MacManes, M.D., Ott, M., Orvis, J., Pochet, N., Strozzi, F., Weeks, N., Westerman, R., William, T., Dewey, C.N., Henschel, R., LeDuc, R.D., Friedman, N., Regev, A., 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat Protoc 8, 1494–1512. https://doi.org/10.1038/nprot.2013.084.

Harkin, G., Hopkinson, H., 2010. Carbamazepine. Pract Diab Int 27, 205–206. https://doi.org/10.1002/pdi.1485.

Harms, L., Frickenhaus, S., Schiffer, M., Mark, F.C., Storch, D., Held, C., Pörtner, H.-O., Lucassen, M., 2014. Gene expression profiling in gills of the great spider crab Hyas araneus in response to ocean acidification and warming. BMC Genom. 15, 789 https://doi.org/10.1186/1471-2164-15-789.

Hawkins, C.A., Sokolova, I.M., 2017. Effects of elevated CO2 levels on subcellular distribution of trace metals (Cd and Cu) in marine bivalves. Aquat. Toxicol. 192, 251–264. https://doi.org/10.1016/j.aquatox.2017.09.028.

Heye, K., Lotz, T., Wick, A., Oehlmann, J., 2019. Interactive effects of biotic and abiotic environmental stressors on carbamazepine toxicity in the non-biting midge *Chironomus riparius*. Water Res. 156, 92–101. https://doi.org/10.1016/j.watres.2019.03.007.

Hoegh-Guldberg, O., Cai, R., Poloczanska, E.S., Brewer, P.G., Sundby, S., Hilmi, K., Fabry, V.J., Jung, S., 2015. The ocean. Climate Change 2014: Impacts, Adaptation and Vulnerability: Part B: Regional Aspects: Working Group II Contribution to the

Fifth Assessment Report of the Intergovernmental Panel on Climate Change, pp. 1655-1734. DOI: https://doi.org/10.1017/CBO9781107415386.010.

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 (1), 44–57. https://doi.org/10.1038/nprot.2008.211.

Italian Legislative Decree n. 26, March 4, 2014. Implementation of Directive 2010/63 / EU on the protection of animals used for scientific purposes, 2014.

Ivanina, A.V., Sokolova, I.M., 2015. Interactive effects of metal pollution and ocean acidification on physiology of marine organisms. Curr. Zool. 61, 653–668. https://doi.org/10.1093/czoolo/61.4.653.

Kang, P., Zhang, W., Chen, X., Yi, X., Song, P., Chang, Y., Zhang, S., Gao, T., Li, C., Li, S., 2018. TRPM2 mediates mitochondria-dependent apoptosis of melanocytes under oxidative stress. Free Radic. Biol. Med. 126, 259–268. https://doi.org/10.1016/j.freeradbiomed.2018.08.022.

Klosterhaus, S.L., Grace, R., Hamilton, M.C., Yee, D., 2013. Method validation and reconnaissance of pharmaceuticals, personal care products, and alkylphenols in surface waters, sediments, and mussels in an urban estuary. Environ. Int. 54, 92–99. https://doi.org/10.1016/j.envint.2013.01.009.

Kroeker, K.J., Kordas, R.L., Crim, R.N., Singh, G.G., 2010. Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecol. Lett. 13, 1419–1434. https://doi.org/10.1111/j.1461-0248.2010.01518.x.

Leprêtre, M., Almunia, C., Armengaud, J., Salvador, A., Geffard, A., Palos-Ladeiro, M., 2019. The immune system of the freshwater zebra mussel, *Dreissena polymorpha*, decrypted by proteogenomics of hemocytes and plasma compartments. J. Proteomics 202, 103366. https://doi.org/10.1016/j.jprot.2019.04.016.

Richards, M., Xu, W., Mallozzi, A., Errera, R.M., Supan, J., 2018. Production of calcium-binding proteins in Crassostrea virginica in response to increased environmental CO_2 concentration. Front. Mar. Sci. 5, 203, 10.3389. https://doi.org/10.3389/fmars.2018.00203.

Maranho, L.A., Baena-Nogueras, R.M., Lara-Martín, P.A., DelValls, T.A., Martín-Díaz, M. L., 2015. Acandidateshort-termtoxicitytestusing *Ampelisca brevicornis* to assess sublethal responses to pharmaceuticals bound to marine sediments. Arch. Environ. Contam. Toxicol. 68, 237–258. https://doi.org/10.1007/s00244-014-0080-0.

Martin-Diaz, L., Franzellitti, S., Buratti, S., Valbonesi, P., Capuzzo, A., Fabbri, E., 2009. Effects of environmental concentrations of the antiepileptic drug carbamazepine on biomarkers and cAMP mediated cell signalling in the mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 94, 177–185. https://doi.org/10.1016/j.aquatox.2009.06.015.

Martínez Bueno, M.J., Boillot, C., Fenet, H., Chiron, S., Casellas, C., Gómez, E., 2013. Fast and easy extraction combined with high resolution-mass spectrometry for residue analysis of two anticonvulsants and their transformation products in marine mussels. J. Chromatogr. A 1305, 27–34. https://doi.org/10.1016/j.chroma.2013.06.071.

Mezzelani, M., Gorbi, S., Da Ros, Z., Fattorini, D., d'Errico, G., Milan, M., Bargelloni, L., Regoli, F., 2016a. Ecotoxicological potential of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in marine organisms: bioavailability, biomarkers and natural occurrence in *Mytilus galloprovincialis*. Mar. Environ. Res. 121, 31–39. https://doi.org/10.1016/j.marenvres.2016.03.005.

Mezzelani, M., Gorbi, S., Fattorini, D., d'Errico, G., Benedetti, M., Milan, M., Bargelloni, L., Regoli, F., 2016b. Transcriptional and cellular effects of non-Steroidal Anti-inflammatory drugs (NSAIDs) in experimentally exposed mussels, *Mytilus galloprovincialis*. Aquat. Toxicol. 180, 306–313. https://doi.org/10.1016/j.aquatox.2016.10.006.

Mezzelani, M., Gorbi, S., Fattorini, D., d'Errico, G., Consolandi, G., Milan, M., Bargelloni, L., Regoli, F., 2018a. Long-term exposure of *Mytilus galloprovincialis* to Diclofenac, Ibuprofen and Ketoprofen: insights into bioavailability, biomarkers and transcriptomic changes. Chemosphere 198, 238–248. https://doi.org/10.1016/j.chemosphere.2018.01.148.

Mezzelani, M., Gorbi, S., Regoli, F., 2018b. Pharmaceuticals in the aquatic environments: evidence of emerged threat and future challenges for marine organisms. Mar. Environ. Res. 140, 41–60. https://doi.org/10.1016/j.marenvres.2018.05.001.

Mezzelani, M., Fattorini, D., Gorbi, S., Nigro, M., Regoli, F., 2020. Human pharmaceuticals in marine mussels: evidence of sneaky hazard along Italian coasts. Mar. Environ. Res. 162, 105137 https://doi.org/10.1016/j.marenvres.2020.105137.

Milan, M., Carraro, L., Fariselli, P., Martino, M.E., Cavalieri, D., Vitali, F., Boffo, L., Patarnello, T., Bargelloni, L., Cardazzo, B., 2018b. Microbiota and environmental stress: how pollution affects microbial communities in Manila clams. Aquatic Toxicology 194, 195–207. https://doi.org/10.1016/j.aquatox.2017.11.019.

Milan, M., Dalla Rovere, G., Smits, M., Ferraresso, S., Pastore, P., Marin, M.G., Patarnello, T., Bargelloni, L., Matozzo, V., 2018a. Ecotoxicological effects of the herbicide glyphosate in non-target aquatic species: Transcriptional responses in the mussel Mytilus galloprovincialis. Environ. Pollut. 237, 442–451. https://doi.org/10.1016/j.envpol.2018.02.049.

Milan, M., Smits, M., Dalla Rovere, G., Iori, S., Zampieri, A., Carraro, L., Martino, C., Papetti, C., Ianni, A., Ferri, N., Iannaccone, M., Patarnello, T., Brunetta, R., Ciofi, C., Grotta, L., Arcangeli, G., Bargelloni, L., Cardazzo, B., Martino, G., 2019. Host-microbiota interactions shed light on mortality events in the striped venus clam Chamelea gallina. Mol. Ecol. 28, 4486–4499. https://doi.org/10.1111/mec.15227.

Miller, T.H., Ng, K.T., Bury, S.T., Bury, N.R., Barron, L.P., 2019. Biomonitoring of pesticides, pharmaceuticals and illicit drugs in a freshwater invertebrate to estimate toxic or effect pressure. Environ. Int. 129, 595–606. https://doi.org/10.1016/j.envint.2019.04.038.

Mizuno, K., Okada, M., Murakami, T., Kamata, A., Zhu, G., Kawata, Y., Wada, K., Kaneko, S., 2000. Effects of carbamazepine on acetylcholine release and metabolism. Epilepsy Res 40, 187–195. https://doi.org/10.1016/s0920-1211(00)00129-7.

Moore, M.N., Allen, J.I., McVeigh, A., 2006. Environmental prognostics: an integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. Mar. Environ. Res. 61, 278–304. https://doi.org/10.1016/j.marenvres.2005.10.005.

Moreira, R., Pereiro, P., Canchaya, C., Posada, D., Figueras, A., Novoa, B., 2015. RNASeq in *Mytilus galloprovincialis*: comparative transcriptomics and expression profiles among different tissues. BMC Genomics 16, 728. https://doi.org/10.1186/s12864-015-1817-5.

Munari, M., Matozzo, V., Chemello, G., Riedl, V., Pastore, P., Badocco, D., Marin, M.G., 2019. Seawater acidification and emerging contaminants: a dangerous marriage for haemocytes of marine bivalves. Environ. Res. 175, 11–21. https://doi.org/10.1016/j.envres.2019.04.032.

Nardi, A., Mincarelli, L.F., Benedetti, M., Fattorini, D., d'Errico, G., Regoli, F., 2017. Indirect effects of climate changes on cadmium bioavailability and biological effects in the Mediterranean mussel *Mytilus galloprovincialis*. Chemosphere 169, 493–502. https://doi.org/10.1016/j.chemosphere.2016.11.093.

Nardi, A., Benedetti, M., Fattorini, D., Regoli, F., 2018a. Oxidative and interactive challenge of cadmium and ocean acidification on the smooth scallop *Flexopecten glaber*. Aquat. Toxicol. 196, 53–60. https://doi.org/10.1016/j.aquatox.2018.01.008.

Nardi, A., Benedetti, M., d'Errico, G., Fattorini, D., Regoli, F., 2018b. Effects of ocean warming and acidification on accumulation and cellular responsiveness to cadmium in mussels *Mytilus galloprovincialis*: Importance of the seasonal status. Aquat. Toxicol. 204, 171–179. https://doi.org/10.1016/j.aquatox.2018.09.009.

Nazıroğlu, M., Demirdaş, A., 2015. Psychiatric disorders and TRP channels: focus on psychotropic drugs. Curr Neuropharmacol. 13 (2), 248–257. https://doi.org/10.2174/1570159x13666150304001606.

Ofoegbu, P.U., Simao, F.C.P., Cruz, A., Mendo, S., Soares, A.M.V.M., Pestana, J.L.T., 2016. Toxicity of tributyltin (TBT) to the freshwater planarian *Schmidtea mediterranea*. Chemosphere 148, 61–67. https://doi.org/10.1016/j.chemosphere.2015.12.131.

Peruzza, L., Shekhar, M.S., Kumar, K.V., Swathi, A., Karthic, K., Hauton, C., Vijayan, K. K., 2019. Temporal changes in transcriptome profile provide insights of White Spot Syndrome Virus infection in *Litopenaeus vannamei*. Sci. Rep. 9, 13509. https://doi.org/10.1038/s41598-019-49836-0.

Pierrot, D., Lewis, E., Wallace, D.W.R., 2006. MS Excel Program Developed for CO2 System Calculations. ORNL/CDIAC-105a. doi: 10.3334/CDIAC/otg.CO2SYS_XLS_CDIAC105a.

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. https://doi.org/10.1016/j.chemosphere.2010.12.064.

Raftopoulou, E.K., Dailianis, S., Dimitriadis, V.K., Kaloyianni, M., 2006. Introduction of cAMP and establishment of neutral lipids alterations as pollution biomarkers using the mussel Mytilus galloprovincialis. Correlation with a battery of biomarkers. Sci. Total Environ. 368, 597–614. https://doi.org/10.1016/j.scitotenv.2006.04.031.

Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar. Environ. Res. 93, 106–117. https://doi.org/10.1016/j.marenvres.2013.07.006.

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 MusselWatch. Mar. Environ. Res. 96, 92–104. https://doi.org/10.1016/j.marenvres.2013.09.016.

Regoli, F., D'Errico, G., Nardi, A., Mezzelani, M., Fattorini, D., Benedetti, M., Di Carlo, M., Pellegrini, D., Gorbi, S., 2019. Application of a Weight Of Evidence approach for monitoring complex environmental scenarios: the case-study of off-shore platforms. Front. Mar. Sci. 6, 377. https://doi.org/10.3389/fmars.2019.00377.

Risso, D., Ngai, J., Speed, T.P., Dudoit, S., 2014. Normalization of RNA-seq data using factor analysis of control genes or samples. Nat. Biotechnol. 32, 896–902. https://doi.org/10.1038/nbt.2931.

Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140. https://doi.org/10.1093/bioinformatics/btp616.

Serra-Compte, A., Maulvault, A.L., Camacho, C., Álvarez-Muñoz, D., Barceló, D., Rodríguez-Mozaz, S., Marques, A., 2018. Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (*Mytilus galloprovincialis*). Environ. Pollut. 236, 824–834. https://doi.org/10.1016/j.envpol.2018.02.018.

Shi, Y., Zhang, Y., Zhao, F., Ruan, H., Huang, H., Luo, L., Li, L., 2014. Acetylcholine serves as a derepressor in Loperamide-induced Opioid-Induced Bowel Dysfunction (OIBD) in zebrafish. Sci Rep 4, 5602.

Siebel, A.M., Rico, E.P., Capiotti, K.M., Piato, A.L., Cusinato, C.T., Franco, T.M.A., Bogo, M.R., Bonan, C.D., 2010. In vitro effects of antiepileptic drugs on acetylcholinesterase and ectonucleo-tidase activities in zebrafish (*Danio rerio*) brain. Toxicol. In Vitro 24, 1279–1284. https://doi.org/10.1016/j.tiv.2010.03.018.

Stillman, J.H., Paganini, A.W., 2015. Biochemical adaptation to ocean acidification. J. Exp. Biol. 218, 1946–1955. https://doi.org/10.1242/jeb.115584.

Suwalsky, M., Mennickent, S., Norris, B., Villena, F., Sotomayor, C.P., 2006. Effects of the antiepileptic drug carbamazepine on human erythrocytes. Toxicol. In Vitro 20, 1363–1369. https://doi.org/10.1016/j.tiv.2006.05.010.

Swiacka, K., Maculewicz, J., Smolarz, K., Szaniawska, A., Caban, M., 2019. Mytilidae as model organisms in the marine ecotoxicology of pharmaceuticals – a review. Environ. Pollut. 254, 113082 https://doi.org/10.1016/j.envpol.2019.113082.

Takahashi, N., Kozai, D., Kobayashi, R., Ebert, M., Mori, Y., 2011. Roles of TRPM2 in oxidative stress. Cell Calcium 50, 279–287. https://doi.org/10.1016/j.ceca.2011.04.006.

Trombini, C., Hampel, M., Blasco, J., 2019. Assessing the effect of human pharmaceuticals (carbamazepine, diclofenac and ibuprofen) on the marine clam *Ruditapes philippinarum*: an integrative and multibiomarker approach. Aquat. Toxicol. 208, 146–156. https://doi.org/10.1016/j.aquatox.2019.01.004.

Wille, K., Kiebooms, J.L., Claessens, M., Rappé, K., Vanden Bussche, J., Noppe, H., Van Praet, N., De Wulf, E., Van Caeter, P., Janssen, C.R., De Brabander, H.F., Vanhaecke, L., 2011. Development of analytical strategies using U-HPLC-MS/MS and LC-ToF-MS for the quantification of micropollutants in marine organisms. Anal. Bioanal. Chem. 400, 1459–1472. https://doi.org/10.1007/s00216-011-4878-6.

Zhang, L., Hui, Y.N., Wang, Y.S., Ma, J.X., Wang, J.B., Ma, L.N., 2011. Calcium overload is associated with lipofuscin formation in human retinal pigment epithelial cells fed with photoreceptor outer segments. Eye 25 (4), 519–527. https://doi.org/10.1038/eye.2011.7.