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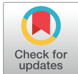
Mixtures of environmental pharmaceuticals in marine organisms: Mechanistic evidence of carbamazepine and valsartan effects on *Mytilus galloprovincialis*



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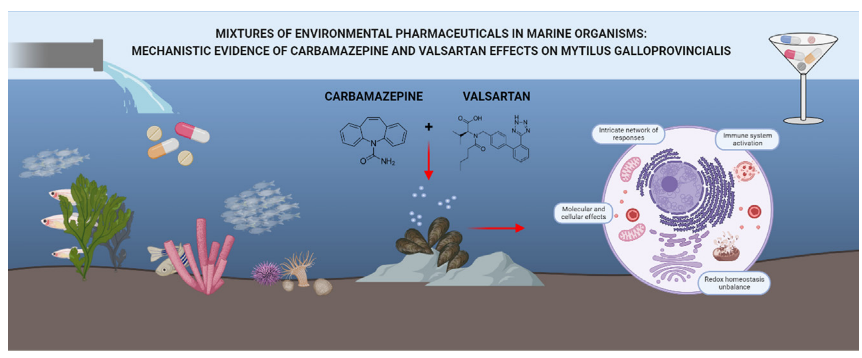
HIGHLIGHTS

- Uptake of drugs from mixtures in *M. galloprovincialis* is modulated by competing mechanisms.
- Carbamazepine (CBZ) exerts a greater cellular reactivity than valsartan (VAL).
- Antagonistic effects of CBZ and VAL were observed at molecular and cellular levels.
- Immunocompetence and redox homeostasis were the most affected pathways.
- Cellular effects of CBZ remained evident after depuration.

GRAPHICAL ABSTRACT



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ABSTRACT

Unravelling the adverse outcomes of pharmaceuticals mixture represents a research priority to characterize the risk for marine ecosystems. The present study investigated, for the first time, the interactions between two of the most largely detected pharmaceuticals in marine species: carbamazepine (CBZ) and valsartan (VAL), elucidating mechanisms that can modulate bioaccumulation, excretion and the onset of toxicity. *Mytilus galloprovincialis* were exposed to environmental levels of CBZ and VAL dosed alone or in combination: measurement of drug bioaccumulation was integrated with changes in the whole transcriptome and responsiveness of various biochemical and cellular biomarkers. Interactive and competing mechanisms between tested drugs were revealed by the much higher CBZ accumulation in mussels exposed to this compound alone, while an opposite trend was observed for VAL. A complex network of responses was observed as variations of gene expression, functional effects on neurotransmission, cell cycle, immune responses and redox homeostasis. The elaboration of results through a quantitative Weight of Evidence model summarized a greater biological reactivity of CBZ compared to VAL and antagonistic interactions between these compounds, resulting in a reduced effect of the antiepileptic when combined with valsartan. Overall, new perspectives are highlighted for a more comprehensive risk assessment of environmental mixtures of pharmaceuticals.

1. Introduction

In recent years, the presence of pharmaceutical residues in aquatic ecosystems has received increasing attention. Wastewater treatment plants represent the main source for their release in surface waters along with industrial and hospital discharges, aquaculture facilities, animal farming, soil runoff, and direct discharge of untreated wastewater (Bagnis et al., 2019;

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Mezzelani and Regoli, 2022). Pharmaceuticals have been detected in several environmental matrices, including coastal water and sediments at concentrations ranging from a few ng to several μg per L or Kg, respectively (Almeida et al., 2021, Madikizela et al., 2020; Mezzelani and Regoli, 2022; Pereira et al., 2015). According to available estimates, >4000 biologically active ingredients, extremely diverse in terms of chemical structures, physical characteristics, and mechanisms of action, can reach natural ecosystems and interact with non-target species causing unknown biological effects, especially in terms of long-term consequences (Mezzelani and Regoli, 2022). Recent data highlighted the capability of marine organisms to accumulate various typologies of pharmaceuticals reaching concentrations up to hundreds ng/g dry weight (d.w.) (Martínez-Morcillo et al., 2020, Swiacka et al., 2019); single active ingredients were documented to affect several molecular and cellular pathways, revealing that type and magnitude of sublethal outcomes are dose- and compound-dependent. However, a key feature when assessing the environmental impact of pharmaceuticals under field conditions, is that organisms are typically exposed to low doses of several co-occurring drugs that can modulate the overall toxicity through different but often overlapping pathways (Mezzelani and Regoli, 2022). Although pharmaceuticals mixtures have been measured in fish and shellfish from the Mediterranean, Red Sea, and Indian Ocean (Ali et al., 2018; Mezzelani and Regoli, 2022; Swiacka et al., 2019; Omar et al., 2019), potential combined effects and adverse biological outcomes resulting from pharmaceuticals interactions have received so far limited attention.

A large-scale investigation on Mediterranean mussels, *Mytilus galloprovincialis,* collected over 4 years from 14 sites along the Adriatic and Tyrrhenian Sea, revealed the antiepileptic carbamazepine (CBZ) and the antihypertensive valsartan (VAL) as the most frequently detected pharmaceuticals, measured in >95 % and 55 % of analyzed samples, respectively (Mezzelani et al., 2020).

CBZ is a first-generation anticonvulsant drug used to treat epilepsy, which ranks fourth in the world's disease burden after tension-type headache, migraine and Alzheimer's disease (Beydoun et al., 2020). Characterized by an estimated worldwide consumption of >1000 tons per year, CBZ is unfortunately well-known for resistance to biotreatments and photodegradation processes, and the consequent persistence in all aquatic compartments including fresh, marine, surface, ground, and even drinking water (Almeida et al., 2021; Zhu et al., 2019). Most of the investigation on CBZ occurrence in aquatic ecosystems was performed in European waters and revealed average levels ranging from < LOQ (limit of quantification) up to hundreds of ng/L, with peaks of 1410 ng/L detected in the east coast of Ireland, close to wastewater outfall pipe (Almeida et al., 2021). In marine biota, CBZ was determined mainly in bivalve species from few coastal systems, with highest concentrations detected in the marine clams from the estuary systems of Ria Formosa (13.2 ng/g dry weight, d.w.) and mussels from the Italian cost (299.7 ng/g d.w. Almeida et al., 2020, 2021; Mezzelani et al., 2020). Therapeutic effects of CBZ in mammals rely on its capability to block the voltage-gated sodium channels, to antagonize the gamma-aminobutyric acid (GABA) receptor, to inhibit glutamate release and the entrance of chloride into cells (Siebel et al., 2010). Fascinating similarities were recently highlighted in modes of action of CBZ between target and nontarget species: laboratory investigations on a large variety of marine invertebrates (e.g. *M. galloprovincialis, Ruditapes philippinarum, Venerupis decussata, Scrobicularia plana, Diopatra neapolitana)* and vertebrates (e.g. *Sparus aurata*) showed the reduction of neuronal excitability, changes in transmembrane transport, modulation of serotonin metabolism, onset of oxidative stress, and neurotoxicity (Mezzelani and Regoli, 2022). Further evidence of embryo toxicity in *Mytilus* spp. and impairment of androgen and estrogen metabolism, allowed to hypothesize that CBZ can act as endocrine disruptor, with potentially deleterious, chronic consequences for reproductive performance and population sustainability (Almeida et al., 2020, 2021; Mezzelani and Regoli, 2022; Oliveira et al., 2017).

VAL is an angiotensin II type-1 receptor blocker belonging to the wide family of Sartans, the most frequently prescribed agents to treat human hypertension, myocardial infarction, heart failure, and coronary/artery diseases (Gallego et al., 2021; Mezzelani and Regoli, 2022). Compared to CBZ, the occurrence of cardiovascular compounds, particularly valsartan, is much less investigated, with few studies reporting levels in coastal areas from <LOQ up to 92 ng/L, and concentrations in marine mussels up to 6.7 ng/g d.w. (Mezzelani et al., 2018, 2020; Zhang et al., 2020). Noteworthy, the ecotoxicological consequences and mechanism of action of antihypertensives in non-target species are quite unexplored, representing a priority research gap that needs to be filled (Gallego et al., 2021; Mezzelani and Regoli, 2022; Zhang et al., 2020).

The present study was aimed to investigate for the first time the interactions between environmental levels of CBZ and VAL providing novel insights on the mechanisms that can modulate bioaccumulation, excretion and onset of sublethal effects in the Mediterranean mussel *M. galloprovincialis*. In this respect, a wide and multidisciplinary approach was undertaken integrating drug bioaccumulation with RNA-sequencing of transcriptomic responses and an extensive panel of biochemical, cellular and histological markers. Such responses, reflecting the main pathways of action, metabolism, and toxicity of selected pharmaceuticals, included impairment of immunological parameters, modulation of lipid metabolism, alteration of antioxidant system and onset of oxidative damages. The biological significance of the overall results was finally summarized through a quantitative Weight Of Evidence model (WOE, Sediqualsoft) that elaborates specific hazard indices based on the number, magnitude and toxicological relevance of the observed responses (Regoli et al., 2019). The present study was expected to demonstrate interactive effects and Mechanism Of Action (MOA) of two of the most frequently detected pharmaceuticals in Mediterranean organisms, thus corroborating the importance of considering mixture toxicity and multiple stressors toward the development of science-based environmental risk assessment.

2. Materials and methods

2.1. Animal collection and experimental plan

Mussels, *Mytilus galloprovincialis* (5.3 ± 0.5 cm shell length), were obtained in June 2020 from a shellfish farm in a reference area of central Adriatic Sea (Regoli et al., 2014). Organisms were acclimatized for 10 days in aquaria with aerated artificial seawater (ASW; Instant Ocean®) at local seasonal environmental conditions of salinity (35 practical salinity units), temperature (23 °C) and pH (8.20). Collection and experimental use of mussels is not subjected to ethical review permissions according to both European and Italian normative (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, 2010; Italian Legislative Decree n. 26, 2014), while monitoring guidelines recommend this species as appropriate bioindicator organism for assessing bioavailability of ecotoxicological effects of environmental pollutants in marine environments (Bocchetti and Regoli, 2006).

Mussels were randomly assigned to eight 20 L tanks each containing 60 organisms and exposed to the four following treatments, in duplicate: CTL, control condition; CBZ, carbamazepine exposure (0.5 μg/L); VAL, valsartan exposure (0.5 μg/L); CBZ + VAL, combined mixture of carbamazepine (0.5 μg/L) and valsartan (0.5 μg/L). After 14 days of exposure, organisms were maintained for additional 14 days in carbamazepine- and valsartan-free ASW, intended as recovery phase from the tested active pharmaceuticals ingredients. The rationale for selecting 14 days for both the exposure and depuration phase was based on background knowledge on physiology and responsiveness of the selected bioindicator species to different typologies of environmental pollutants (e.g. Gonzalez-Rey et al., 2014; Gonzalez-Rey and Bebianno, 2014; Regoli et al., 2014; Mezzelani et al., 2021; Nardi et al., 2022): the period of 14 days is intended as a relative short-term time enabling *M. galloprovincialis* (i) to biologically react to tested compounds avoiding the potential activation of adaptive mechanisms, (ii) recover from or (iii) highlight the persistence of a stressful condition. The exposure doses of CBZ and VAL are environmentally realistic and

typically found in coastal areas (Birch et al., 2015; Freitas et al., 2016; Gaw et al., 2014; Mezzelani et al., 2018). Stock solutions of carbamazepine and valsartan (Sigma Aldrich) were 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. Water was changed every other day and, during the exposure phase, pharmaceuticals were re-dosed. Mussels were fed 12 h prior the water change with 500 μL of a commercial mixture of zooplankton (Brightwell Zooplanktos-S, size range 50–300 μm) for filter-feeding organisms, according to manufacturer indications. From each experimental condition (CTL, CBZ, VAL, CBZ + VAL), at each experimental phase (Exposure, 14 days and Recovery, 28 days), 25 individuals were sampled from each duplicate tank, for a total of 50 organisms, randomly mixed then dissected and pooled for bioaccumulation, transcriptomic and biomarker analyses as follow: for chemical analyses, 3 pools each constituted by the whole tissues of 5 organisms were collected and stored at −20 °C. For transcriptomic and biochemical analyses, gills, digestive glands and haemolymph 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. Aliquots of haemolymph were immediately processed for in vivo analysis of lysosomal membrane stability in haemocytes, granulocytes-hyalinocytes ratio and phagocytosis rate, while additional aliquots of haemolymph were fixed in Carnoy's solution (3:1 methanol, acetic acid) for the evaluation of micronuclei frequency. Digestive glands from additional 11 organisms were excised, flash frozen separately and maintained at −80 °C for histological analyses.

2.2. Chemical analyses

Protocols for measuring tissues concentrations of tested pharmaceuticals in exposed mussel by high performance liquid chromatography, HPLC, were recently validated (Mezzelani et al., 2018, 2020) and full details are given in Supplementary materials (SM1). Briefly, stock solutions of Carbamazepine and Valsartan (1 mg mL^{-1}) were prepared in methanol and stored in amber vials (10 mL). Working solutions were prepared daily in ultra-pure water, as required. All solutions were stored at +4 °C and in the dark to reduce possible degradation. Acetic acid 0.1 % pH = 3.26 (Buffer 1), was used as homogenization and extraction buffer. About 3 g of wet tissues were homogenized in 5 mL of buffer at room temperature for 20 min. After centrifugation at 4500 ×*g* for 30 min, samples were purified by Solid Phase Extraction (SPE) with reversed-phase tubes (Discovery DSC-18, 1 g × 6 mL, Supelco, Bellefonte, Pennsylvania, USA). SPE tubes were conditioned with 6 mL of methanol, followed by 18 mL of ultra-pure water. Samples were diluted (1:1) with ultra-pure water and loaded onto the SPE cartridges; after washing with 12 mL of potassium bicarbonate KHCO3 and 6 mL of ultra-pure water, analytes were eluted and recovered using 2 mL of Methanol and Acetic Acid (0.1 %). Obtained samples were filtered using PhenexTM-RC membrane (Regenerated Cellulose/Polypropylene 0.45 μm, 15 mm syringe filters, Phenomenex, US) and then centrifuged again at 12,000 ×*g* for 20 min. Analytical detection of extracted pharmaceuticals was performed by High Performance Liquid Chromatography, with fluorimetric and diode array detectors DAD (Agilent Infinity 1260 series). Chromatographic separations of CBZ and VAL was performed on a Kinetex column (C18,5 μm, 150 mm length, 4.6 mmID, Phenomenex, US), equipped with a security guard column (C18, 5 μm, 4 mm length, 2.0 mmID, Phenomenex, US). A mobile phase composed by ultra-pure water (26 %), acetonitrile (42 %) and Buffer 1 (32 %) was used under isocratic condition. DAD was used for monitoring the spectra from 190 nm to 350 nm, and the signal for CBZ was obtained at 286 nm, while analytical detection of VAL was obtained by fluorimetric detector with excitation/emission wavelengths at 205/380 nm. CBZ and VAL concentrations were quantified by comparison with signals of pure standard solutions. Due to the lack of appropriate Certified Standard Reference Materials (SRMs), CBZ and VAL recovery was estimated on samples of control mussels ($n = 10$) spiked with various concentrations of investigated molecules to assess the reproducibility, quality assurance and quality control of analytical procedures. The specifically developed conditions for sample preparation, purification and concentration, allowed a recovery yield always ≥98 % (CV < 5 %, $n = 10$), with an instrumental limit of detection (LOD) in mussel tissues of 1.03 and 0.48 ng/g dry weight (d.w.) for CBZ and VAL respectively. All those values always ensure an appropriate analytical accuracy.

2.3. Transcriptomic analyses: RNA extraction, RNAseq library preparation, sequencing and data analysis

For each treatment total RNA was extracted after 14 and 28 days with a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions from 5 pools of digestive glands of (each composed by 3 randomly selected individuals). Library preparation for gene expression analysis was performed using QuantSeq 3′ mRNA-Seq Library Prep Kit FWD Lexogen for Illumina. The library pools were sequenced on Illumina Novaseq 6000 (CRIBI; University of Padova) with a single-end 75 bp setup obtaining a total of 245′566′382 reads (sequences available in NCBI SRA; https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA851736). Gene expression profiles were explored through at different levels: i) principal component analysis (PCA) as unsupervised method performed considering all samples within each sampling time; ii) pairwise comparisons between CTL and each treatment to identify differentially expressed genes; iii) functional analyses through Gene Set Enrichment Analysis (GSEA) to determine whether a priori defined set of genes representing different molecular pathways shows significant variations following drugs exposure.

In detail, input reads were quality checked with FastQC/v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and only high-quality reads were kept after trimming with BBDuk (program specific options were taken from the Lexogen's website at: https://www.lexogen.com/quantseq-data-analysis/) of the suite BBTools (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/). High-quality reads were subsequently mapped against the reference transcriptome from Moreira et al., 2015, by using Kallisto/v0.46.2 with options "–single –fragment-length 100 –sd 40". The "abundance_estimates_to_matrix.pl" script from the Trinity suite was used to generate the count table. Raw read counts were then imported into R/v4.1.0 (R Core Team, 2014) and filtered: we removed contigs with <1 count per million (CPM) in at least 24 libraries (out of 40), which would contribute to background noise (Peruzza et al., 2021; Pradhan et al., 2020). After removal of transcripts with low expression levels, libraries were split according to exposure time (i.e. day 14 and day 28) and a separate analysis was run for each exposure time. Filtered reads were normalized using the RUVs function (with parameter "k = 7" for samples of day 14 and with k = 8 for samples of day 28) from the RUVSeq/v1.26 library (Gerstner et al., 2016; Verma et al., 2021). For each sampling day (i.e. day 14 and 28) a Principal Component Analysis plot (PCA) was generated on the normalized data by using the plotPCA function of the RUVSeq package. Then normalized counts were also used to perform pairwise comparisons with edgeR/v3.34.0 (Robinson et al., 2010). Genes with FDR < 0.05 and FC ≥ |2 were deemed differentially expressed. Functional annotation of the reference transcriptome was performed by Blastx similarity search on Swissprot (Uniprot), *Homo sapiens* protein Ensembl database, *Danio rerio* protein Ensembl database and *Crassostrea gigas* protein Ensembl database (Evalue <0.0001). A Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) was performed to extract biological insight from RNA-seq files to reveal coordinated changes in gene expression data. GSEA was performed using the ClusterProfiler/v4.0.2 package (Wu et al., 2021) against the GO, KEGG and Reactome databases. An FDR cutoff <0.1 was deemed significant.

2.4. Biomarkers analyses

Standardized protocols (Bocchetti and Regoli, 2006; Mezzelani et al., 2021) were used to analyse biomarkers and full methodological details are given in Supplementary Material 1 (SM1). Lysosomal membrane stability (NRRT) was evaluated after the haemocytes incubation on a glass slide

with a freshly prepared Neutral Red (NR) working solution and microscopically examined at 20 min intervals to determine the time at which 50 % of cells had lost into the cytosol the dye previously taken up by lysosomes. For the analysis of granulocytes-hyalinocytes ratio, aliquot of haemolymph was dispersed on a glass slide and, after drying, fixed in Beker's fixative. The slides were washed with water and stained with May-Grunwald Giemsa before mounting in Eukitt® and observed with a light microscope (1000 ×). Phagocytosis capacity assay was microscopically evaluated in haemolymph incubated for 2 h with Fluorescein-labelled Zymosan A bioparticles (Invitrogen) added at 10:1 target:haemocyte ratio; phagocytosis was expressed as percentage of cells that internalized at least 3 fluorescent particles. Acetylcholinesterase activity (AChE) was spectrophotometrically assayed in mussels haemolymph (centrifuged at 3000 ×*g* for 5 min) and gills (homogenized in 0.1 M Tris-HCl buffer pH 7.2, 0.25 M sucrose and centrifuged at 10,000 ×*g* for 10 min). Obtained supernatants were spectrophotometrically assayed by the Ellman's reaction at 18 ± 1 °C. The DNA integrity was evaluated chromosomal level by the micronucleus test Micronuclei (MN) frequency was measured in haemocytes, fixed in Carnoy's solution, dispersed on glass slides and stained with the fluorescent dye 40,6-diamidino-2-phenylindole (DAPI) at 100 ng mL^{-1}. For each specimen, 2000 cells with preserved cytoplasm were scored for the presence of micronuclei. For the activity of Acyl CoA oxidase (ACOX) samples of digestive gland were homogenized in 1 mM sodium bicarbonate buffer (pH 7.6) containing 1 mM EDTA, 0.1 % ethanol, 0.01 % Triton X-100 and centrifuged at 500 ×*g* for 15 min at 4 °C. The H_2O_2 production was measured in a coupled assay by following the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalyzed by an exogenous horseradish peroxidase (HRP). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF-DA, 40 μM sodium azide, 0.01 % Triton X-100, 1.2 U/mL HRP in a final volume of 1 mL. After a pre-incubation at 25 °C for 5 min in the dark with an appropriate volume of sample, reactions were started adding the substrate Palmitoyl-CoA, at final concentrations of 30 μM; readings were carried out against a blank without the substrate at 502 nm. Lipofuscin and neutral lipids content were determined on duplicate cryostat sections (8 μm thick) of digestive gland. Slides were fixed in Beker's fixative and stained by Schmrol reaction and Oil red O (ORO) respectively before mounting in glycerol gelatin. For both lipofuscin and neutral lipids, five measurements were made on digestive tubules of each section (10 mussels, two sections for mussel). Quantification of staining intensity was performed with Image-Pro® Plus 6.2 Analysis Software and then normalized to the area of digestive tubules. For enzymatic antioxidants, samples of digestive gland were homogenized in 100 mM K-phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mg/mL bacitracin, 0.008 TIU/mL aprotinin, 1 mg/mL leupeptin, 0.5 mg/mL pepstatin, NaCl 2.5 %, and centrifuged at 110,000 ×*g* for 1 h at 4 °C. Measurements were made with spectrophotometer at a constant temperature of 18 °C. Catalase (CAT) was measured by the decrease in absorbance at 240 nm due to the consumption of 12 mM H_2O_2 in 100 mM K-phosphate buffer pH 7.0. Glutathione reductase (GR) was determined at 340 nm, from NADPH oxidation during the reduction of 1 mM GSSG in 100 mM K-phosphate buffer pH 7.0 and 60 mM NADPH. Glutathione peroxidases (GPx) activities were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to convert the formed GSSG to its reduced form (GSH). The decrease of absorbance was monitored at 340 nm in 100 mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium azide (NaN3) (for hydrogen peroxide assay), 2 mM GSH, 1 unit glutathione reductase, 0.24 mM NADPH, and 0.5 mM hydrogen peroxide or 0.8 mM cumene hydroperoxide as substrates respectively for the Se-dependent and for the sum of Se-dependent and Se-independent forms. Glutathione S-transferases (GST) were determined at 340 nm using 1.5 mM 1-chloro-2,4-dinitrobenzene as substrate (CDNB) and 1 mM GSH, in 100 mM K-phosphate buffer pH 6.5. Total glutathione was enzymatically assayed in supernatant samples obtained after the homogenization in 5 % sulfosalicylic acid with 4 mM EDTA, maintained for 45 min on ice and centrifuged at 37,000 ×*g* for 15 min. The Total Oxyradical Scavenging Capacity (TOSC) was measured in mussels digestive glands homogenized as previously reported for enzymatic antioxidants, without PMSF in the homogenization buffer. The artificially generated radicals (ROO•, •OH) were obtained from the thermal homolysis of 2-2-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP), the iron (plus ascorbate)-driven Fenton reaction, respectively. The absorption of artificially generated oxyradicals by cellular antioxidants was measured by the quantification of inhibited oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas. Ethylene formation was determined by gas-chromatographic analyses and TOSC values were quantified from the equation: $TOSC = 100 - (\int SA / \int CA \times 100)$, where ∫SA and ∫CA are the integrated areas calculated under the kinetic curve produced during the reaction course for respective sample (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay and determined by the Lowry method with Bovine Serum Albumin (BSA) as standard.

2.5. Statistical analyses

Statistical analyses for 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), with appropriate mathematical transformation if necessary. Analysis of variance was applied to test differences between treatments and exposure days (level of significance at $p < 0.001$), the Student Newman-Keuls test, (SNK) was used for post-hoc comparison between means of values ($n = 5$).

For each experimental treatment and time, the results on bioaccumulation and biomarkers analyses were further elaborated through a quantitative Weight Of Evidence model (WOE, Sediqualsoft) that provides synthetic hazard indices for each typology of data (or Line of Evidence, LOE) before the final integration (Regoli et al., 2019). Independent elaborations procedures were applied to LOE on bioaccumulation and biomarkers. For bioavailability hazard the model considered the fold increase and statistical significance of pharmaceuticals accumulation in exposed organisms (Piva et al., 2011); the elaboration of cellular responses is based on a specifically developed algorithm which, for every analyzed biomarker, evaluate the magnitude of observed variation in comparison to a specific threshold, the toxicological relevance (weight) of biological endpoint and the statistical significance of the difference in respect to controls. After normalization of indices to a common scale, individual hazard indices were integrated through a classical weight of evidence approach, and level of risk assigned to 1 of 5 classes, from Absent to Severe. Whole calculations, detailed flow-charts, rationale for weights, thresholds and expert judgements have been previously described in detail (Regoli et al., 2019) and reported in SM1.

3. Results

Results on pharmaceuticals accumulation in mussel tissues are shown in Table 1. After 14 days of exposure a significant increase in tissue concentrations of CBZ and VAL was observed in organisms treated with individual drugs and in combination. Remarkably higher values of CBZ were measured in mussels exposed to CBZ alone compared to those co-exposed to the mixture; conversely, a greater increase of VAL was reported in mixture-exposed organisms compared to those treated with VAL alone. After 14 days of depuration, levels of pharmaceuticals were below LOD in all experimental conditions.

Results of Principal Component Analyses (PCA) on gene expression profiles after 14 days of exposure and at the end of the depuration period (day 28), are shown in Fig. 1. After 14 days (Fig. 1A), the first axis highlighted a clear separation of gene expression profiles in CBZ exposed mussel explaining 12.8 % of the variation, while the second axis (10.51 % of variance) discriminated CBZ + VAL exposed mussels from CTL and VAL treated organisms. A different scenario appeared after 14 days of depuration (Fig. 1B), with the separation along the X-axis of mussels

Table 1
Concentration at different sampling times of Carbamazepine and Valsartan in the whole tissues of *M. galloprovincialis* exposed to experimental conditions. Data given in ng/g dry weight (mean values ± standard deviations, $n = 3$). CTL Control; CBZ Carbamazepine; VAL Valsartan; CBZ+ VAL Carbamazepine + Valsartan.

	CBZ		VAL	
	Day 14	Day 28	Day 14	Day 28
CTL	<1.03	<1.03	<0.48	<0.48
CBZ	95.21 ± 31.80	<1.03	<0.48	<0.48
VAL	<1.03	<1.03	0.536 ± 0.041	<0.48
CBZ + VAL	23.13 ± 0.961	<1.03	0.983 ± 0.320	<0.48

Table 2
Number of differentially expressed (DEGs) identified at 14 and 28 days for each treatment. Number of up- and down-regulated genes in each exposed group compared to control group (CTL) are also reported, as well as the number of genes commonly found differentially expressed at day 14 and day 28 for each treatment.

	CBZ		VAL		CBZ + VAL	
	Day 14	Day 28	Day 14	Day 28	Day 14	Day 28
DEGs	186	27	45	27	45	125
Up-regulated↑	67	6	16	6	31	12
Down-regulated↓	119	21	29	21	14	113
Common at Day 14 and Day 28	3		1		2	

exposed to CBZ + VAL (12.55 % of variance), and a weaker separation of the other treatments observed along the Y-axis (11.33 % of variance). Pairwise comparisons between CTL and CBZ exposed mussels revealed the highest number of DEGs (186) after 14 days, while both VAL and CBZ + VAL exposed mussels revealed a total of 45 DEGs (p-value ≤ 0.05, FC > 2; Table 2). At the end of the depuration phase, the number of DEGs in CBZ and VAL exposed mussels decreased to a total of 27 DEGs in both treatments, while an opposite trend has been observed in CBZ + VAL exposed mussels with an increase to a total of 125 DEGs. Only a few common genes were differentially expressed after the exposure and depuration periods (3, 1 and 2 in CBZ, VAL and CBZ + VAL treatments, respectively). The whole lists of DEGs, is reported in Supplementary Material 2, 3 (SM2–3) and briefly discussed in SM1.

After 14 days of CBZ exposure, GSEA revealed a conspicuous number of significant terms (SM2). Among the significantly enriched KEGG pathways, “cell cycle”, “DNA replication”, “Nucleotide excision repair” and “ribosome” were up-regulated. Similarly, the analyses of REACTOME and GO gene sets showed several up-regulated terms related to cell cycle regulation and “DNA repair” among the most enriched terms. In addition, up-regulation of “muscle contraction”, “smooth muscle contraction”, “regulation of apoptosis”, “epigenetic regulation of gene expression”, and down-regulation of “Neurotransmitter release cycle”, “Death Receptor Signalling” and “defense response to virus” were also found. At day 28, after the depuration period, GSEA confirmed the results obtained through pairwise comparisons with few significantly enriched terms, represented by the up-regulated REACTOME terms “Signal Transduction” and “Hedgehog ‘off’ state” (up-regulated also at 14 days), and the down-regulated GO term “positive regulation of lamellipodium organization”.

Exposure to VAL caused weaker transcriptional responses compared to CBZ (SM1–3). Among others, GSEA highlighted a unique significantly enriched term represented by “ubiquitin-protein transferase regulator activity”, supported also by the up-regulation of *Ubiquitin-conjugating enzyme E2 L3* (*UB2L3*). After depuration, GSEA, revealed the enriched terms “focal adhesion”, “PI3K-Akt signaling pathway”, “ErbB signaling pathway”, “Toll-like receptor signaling pathway” and “NF-kappa B signaling pathway”.

After the combined exposure to CBZ + VAL, several enriched terms were observed, including the down-regulated KEGG pathways “metabolic process”, “phagosome”, “focal adhesion” and “autophagy”. Similarly, analyses of REACTOME terms showed the down-regulation of “cell-cell communication”, “signal transduction”, “innate immune system”, “metabolism of lipids”, “Golgi associated vesicle biogenesis”, “COPI-mediated anterograde transport”, “peroxisomal protein import”, “signaling by Rho GTPases” and “RAC1 GTPase cycle”. After the depuration period from VAL + CBZ (28 days), a total of five down-regulated GO enriched terms, all related to actin filament and stress fiber, were found. Potential disruption of immune response, inflammation, cell-cycle and apoptosis regulation after the depuration period were also suggested by several differentially expressed genes. Among others, down-regulation of putative *Claspin* (*CLSPN)* should be highlighted.

Results on biochemical and cellular alterations of immunological biomarkers, revealed a significant reduction of the lysosomal membrane stability in all exposed organisms both at day 14 and 28, without differences between compounds dosed alone or in mixture (Fig. 2A). Granulocyte/hyalinocyte ratio did not exhibit any variation during the exposure phase (day 14), while a significant increase compared to CTL was observed for all treatments at the end of the depuration phase (day 28), especially for mussels exposed to CBZ alone (Fig. 2B). A slight, not significant, inhibition of the phagocytosis rate was observed in mussels exposed to CBZ and VAL dosed alone at day 14 (Fig. 2C), while a significant increment in the micronuclei frequency was documented at day 14 for mussels exposed only to VAL (Fig. 2D); this effect was not elicited in mixture-exposed organisms and, at the end of the recovery period (day 28), micronuclei levels from all treatments were comparable to CTL (Fig. 2D).

The activity of acetylcholinesterase was significantly induced at day 14 in organisms exposed to VAL, while at day 28 a marked inhibition was observed in organisms exposed to the mixture (Fig. 3A). Average higher values of the acyl CoA oxidase activity were measured in all treatments at day 14, especially in VAL-exposed mussels; although not significant, a similar trend was also observed at day 28 (Fig. 3B). Histochemical analyses revealed a significant decrease of lipofuscin content in mussels exposed to CBZ and VAL dosed alone, while the mixture treatment caused an

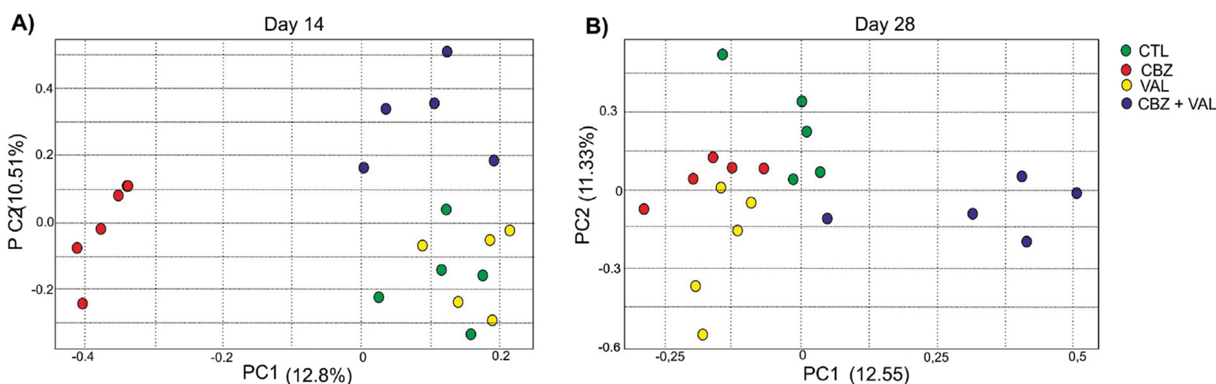

Fig. 1. PCA of the entire set of genes considered for gene expression analyses and after 14 days of exposure and after the end of the depuration phase.

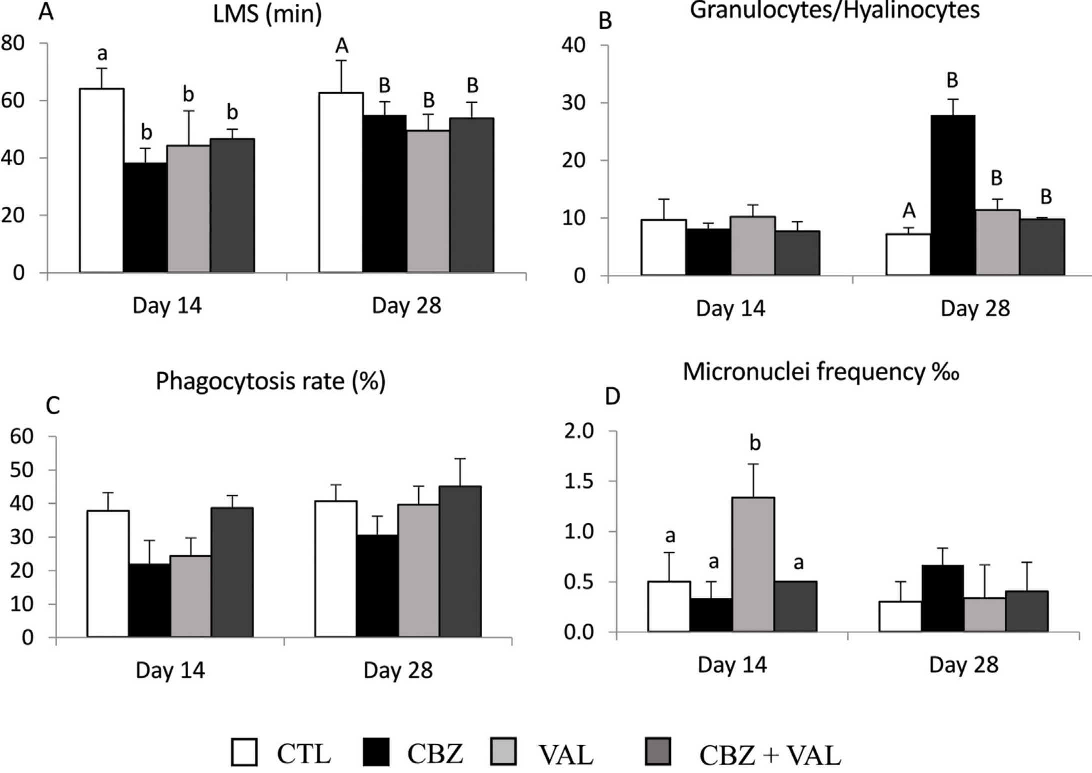

Fig. 2. Lysosomal membrane stability, LMS (A), granulocytes/hyalinocytes ratio (B), phagocytosis rate (C), micronuclei frequency (D) in haemocytes of mussels exposed to various treatments. Data are given as mean values ± Standard Error of the Mean (n = 5). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 14; capital letters are used to highlight significant differences between treatments at day 28. CTL Control; CBZ Carbamazepine; VAL Valsartan; CBZ+ VAL Carbamazepine + Valsartan.

antagonistic interaction with lipofuscin levels comparable to CTL; at a lower extent, a similar pattern of variation was observed at day 28, after 14 days of depuration (Fig. 3C). An increase in mean levels of neutral lipids was measured in VAL and mixture-exposed organisms (VAL and VAL + CBZ) both at day 14 and 28 (Fig. 3D).

Among antioxidant defenses, catalase activity showed a significant induction in organisms exposed to CBZ at day 14 and in VAL- and mixture- treated mussels after the end of the depuration phase (day 28, Fig. 4A). Glutathione S-transferase was significantly increased by CBZ and mixture exposure at day 14 and in VAL- and mixture- exposed mussels at day 28 (Fig. 4B). The activity of glutathione reductase was induced in mussels exposed to CBZ and VAL alone at day 14 and 28, respectively, while no significant variations occurred when pharmaceuticals were combined (Fig. 4C). In all treatments, no changes were observed for levels of total glutathione and glutathione peroxidases (both Se-dependent and the sum of Se-dependent and Se-independent forms) (Fig. 4D-F). The limited prooxidant effects induced by CBZ, VAL and their mixture on exposed mussels were paralleled by the lack of variations of the total oxyradical scavenging capacity toward either peroxyl (ROO•) or hydroxyl (•OH) radicals (Fig. 4G-H).

Synthetic hazard indices for all experimental treatments and times of exposure were provided by the weighted elaboration (WOE) of the LOE on bioaccumulation and the LOE on biomarkers (Fig. 5). After 14 days of exposure, the WOE index revealed a “MAJOR” risk for organisms exposed to CBZ alone, and “SLIGHT” for mussels treated with VAL and the mixture (CBZ + VAL). It is worth to note that both pharmaceuticals determined a different level of hazard on bioaccumulation and cellular responses when dosed alone or in combination. At the end of the recovery phase (day 28), an overall “SLIGHT” level of WOE risk was elaborated for organisms exposed to CBZ which, despite not detectable tissue levels of the drug, still maintained a “Moderate” cellular hazard. An “ABSENT” level of WOE risk was assigned to both VAL and the mixture (CBZ + VAL) at the end of the recovery phase due to the lack of accumulation and only “Slight” cellular hazard (Fig. 5).

4. Discussions

Unravelling the adverse outcomes of environmental pharmaceuticals has recently emerged as a research priority to characterize risk and fate of these ubiquitous compounds.

The environmental relevance of the persistent behaviour of CBZ (Almeida et al., 2018a, 2018b, 2020, 2021; Miller et al., 2019; Mezzelani et al., 2021) was further enhanced by our results which confirmed the capability of *M. galloprovincialis* to accumulate this drug. Interestingly, results of chemical analyses highlighted for the first time a remarkable difference in CBZ concentrations, much higher in mussels exposed to this compound alone compared to those treated with the mixture. An opposite trend was observed for VAL which was accumulated at higher levels in co-exposed mussels compared to those treated with VAL alone. Although the modulation of uptake processes by pharmaceuticals mixtures is almost unexplored in mussels, our results suggest interactive effects and competing mechanisms between the two investigated drugs, resulting in lowered CBZ and enhanced VAL uptake when present together.

The mechanistic explanation for the lowered accumulation of CBZ in the mixture could be related to the fact that both compounds compete for the same cellular channels/transporters and, when simultaneously present, the greater affinity of VAL would determine a lower CBZ uptake (Mezzelani and Regoli, 2022). At the same time, the greater accumulation of VAL when dosed in combination can rely on the inhibitory effect of CBZ on ATP-binding cassette (ABC) transporter P-glycoprotein (P-gp) of *M. galloprovincialis* (Fabbri, 2015). As a key component of the multixenobiotic resistance mechanism, P-gp influences the transmembrane drug efflux and elimination allowing to hypothesize that when cellular efficiency in pumping out is reduced by CBZ (Palleria et al., 2013), the consequent accumulation of VAL is enhanced.

At the end of the depuration phase, levels of both pharmaceuticals were below the limit of detection in all treatments suggesting that biotransformation processes may allow to eliminate drugs (Serra-Compte et al., 2018;

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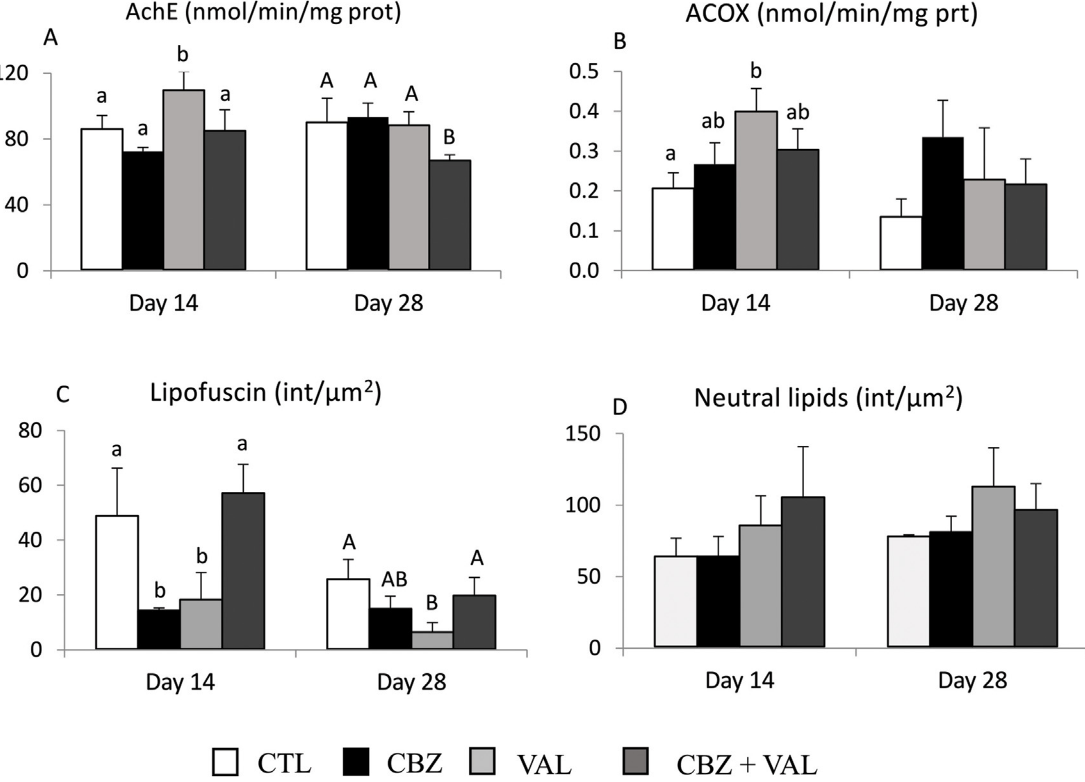

Fig. 3. Acetylcholinesterase activity (A), Acyl Co-A oxidase activity (B), Lipofuscin (C) and neutral lipids (D) content in *M. galloprovincialis* exposed to tested experimental conditions. Data are given as mean values ± Standard deviation ($n = 5$). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 14; capital letters are used to highlight significant differences between treatments at day 28. CTL Control; CBZ Carbamazepine; VAL Valsartan; CBZ+ VAL Carbamazepine + Valsartan.

Mezzelani and Regoli, 2022). Drug metabolism did not appear to be transcriptionally modulated, at cellular level, but GST activity was induced in all treatments at the end of both exposure and depuration phases, highlighting the activation of biotransformation/excretion pathways, and suggesting phase-II conjugation reactions as a key process for pharmaceuticals metabolism in marine mussels. Although the activation of rudimental biotransformation in *M. galloprovincialis* exposed to CBZ has already been highlighted (Mezzelani et al., 2021; Nardi et al., 2022), the present study provided novel insights on metabolisms of VAL, which is largely excreted as unchanged or minimally metabolized compound in target species (Nakashima et al., 2005).

Interactive and competing mechanisms between CBZ and VAL in mussels are further supported by the wide panel responses measured at molecular, biochemical and cellular levels. The transcriptional profile, highlighted a higher number of pathways significantly modulated by CBZ alone compared to both VAL and mixture treatments, suggesting a greater cellular reactivity of the antiepileptic drug in promoting the early onset of molecular alterations, in agreement with insights on bioaccumulation.

Among the main outcomes of CBZ alone, the alteration of neurotransmission should be mentioned at both transcriptional and functional level. The MoA of CBZ relies on modulation of the voltage-gated sodium channels, inhibition of action potentials and decrease of synaptic transmission (Maan et al., 2022). These mechanisms may explain the down-regulation of "neurotransmitter release cycle" among significant REACTOME molecular pathways in mussels exposed to the antiepileptic alone. Previous studies highlighted significant responsiveness of neurotransmission both at molecular and cellular level, with in mRNA transcription of several genes related to neurotransmission, immunity and biomineralization modulation of the *GABA* receptor gene in *Ruditapes philippinarum* and the inhibition of AChE in *M. galloprovicialis*, *Cerastoderma edule* and *Carassius carassius* exposed from 1 to 10 µg/L of CBZ, (Almeida et al., 2018a; Brandts et al., 2018; Jaouani et al., 2021; Mezzelani et al., 2021; Nardi et al., 2022; Nkoom et al., 2020). In the present study, the lack of significant changes in the AChE, might reflect a different time-course of activation of this response between transcriptional and catalytic levels, or a limited effect of CBZ when dosed at <1 µg/L. On the other hand, this enzyme was significantly induced by VAL: although specifically designed to block angiotensin II type-1 receptor, activating a complex cascade of responses ending up with the modulation of cardiac physiology, VAL showed the capability to interact with neuronal mechanisms, eliciting neuroprotective properties in target species (Yang et al., 2014). The exact mechanism of this effect has not been properly investigated and remains almost unexplored in marine organisms (Zhang et al., 2020) but the modulation of AChE activity in our study may represent a first hint on VAL's behaviour in non-target species.

Exposure to CBZ caused significant transcriptional changes of several pathways related to cell cycle, immune response and redox homeostasis. Immunological and oxidative responses, at a lower extent were also modulated in mussels exposed to VAL and mixture of CBZ + VAL, suggesting that independently from their specific mechanism of action, both these drugs can target the same molecular pathways. Although non-synchronous responses between transcriptional and functional effects have been often reported after laboratory exposures (Regoli and Giuliani, 2014), in the present study the transcriptional modulation of immune system, cell cycle and oxidative pathway was paralleled by the onset of related alterations at cellular level. Among these, the significant loss of lysosomal membrane integrity observed in all treatments and the inhibition of phagocytosis in organisms exposed to CBZ and VAL, confirm the capability of tested drugs to lower haemocytes functionality thus impairing mussel's immunocompetence. Previous studies hypothesized immunosuppression as a side effect of CBZ both in target and non-target species, since the modulation of ion channels and GABA receptors in immune cells can provoke impairment of membrane phospholipids leading to multiple cascades of biochemical reactions and cytokines dysregulation (Himmerich et al., 2013; Mezzelani and Regoli, 2022). Conversely, to our knowledge this is the first study revealing

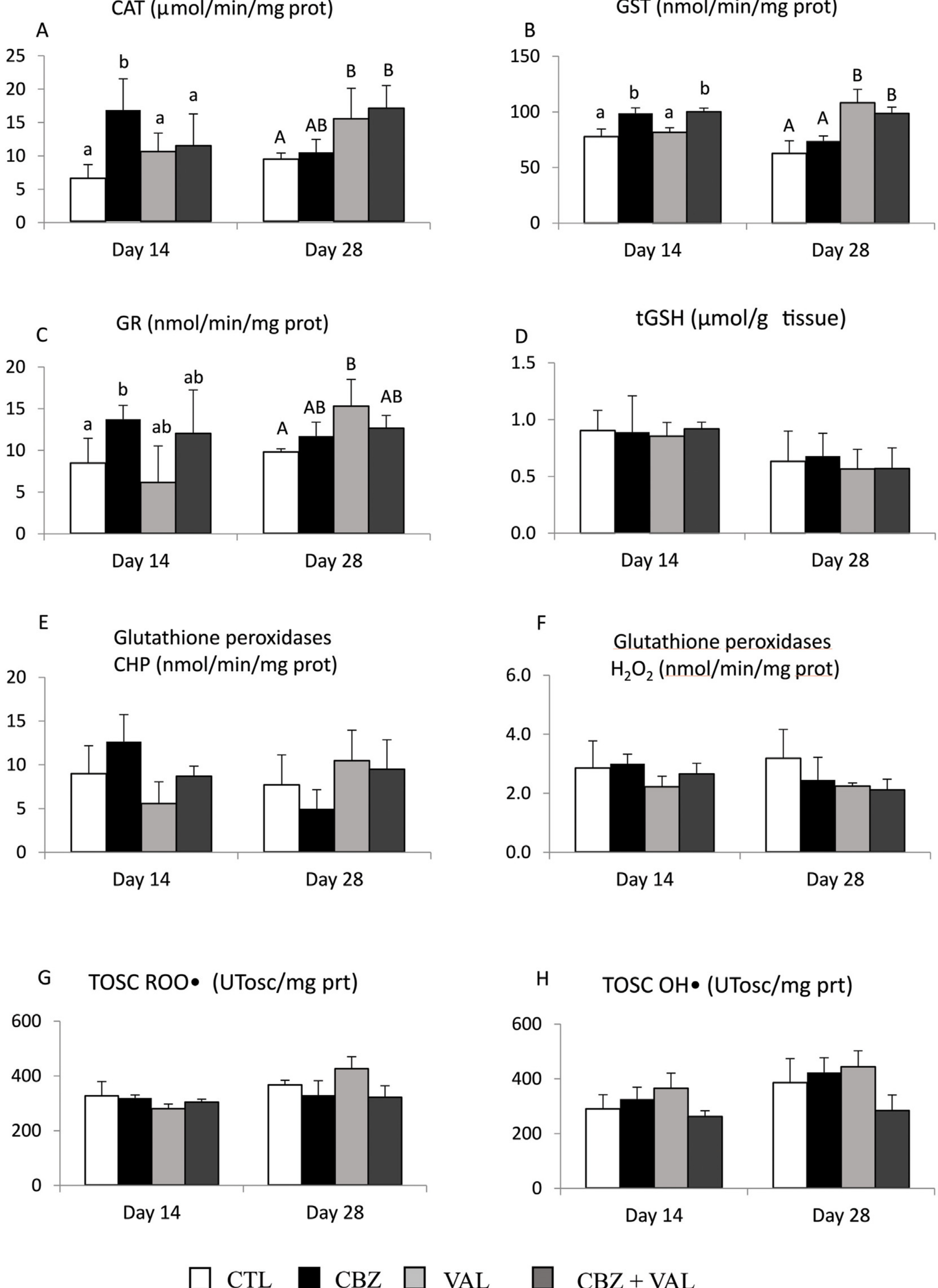

Fig. 4. Antioxidant defenses biomarkers in digestive glands of mussels exposed to various treatments. CAT: catalase (A), GST: glutathione S-transferase (B), GR: glutathione reductase (C), tGSH: total glutathione (D), Glutathione peroxidases CHP: sum of Se-dependent and Se-independent glutathione peroxidases (E), Glutathione peroxidases H_2O_2: Se-dependent glutathione peroxidases (F), TOSC ROO•: total oxyradical scavenging capacity toward peroxyl radical (G), TOSC HO•: total oxyradical scavenging capacity toward hydroxyl radical (H). Data are given as mean values ± Standard deviation (n = 5). Different letters indicate significant differences between group of means: lower case letters are used to highlight significant differences between treatments at day 14; capital letters are used to highlight significant differences between treatments at day 28. CTL Control; CBZ Carbamazepine; VAL Valsartan; CBZ + VAL Carbamazepine + Valsartan.

that also VAL affects the cell mediated immunity in marine organisms as suggested also by the transcriptional changes occurring in Toll-like receptor and NF-kappa B signaling pathways after depuration period.

Oxidative metabolism revealed the significant induction of CAT, GR and a trend for higher activity of GPX in organisms exposed to CBZ alone suggesting a certain influence on redox homeostasis. This evidence was supported at transcriptional level by the modulation of genes encoding phase I-II related proteins, sulfotransferase family and heat shock protein (*CYP1A1*, *SULT1C4*, *SACS*, *HSP12B* and *HSP12A*). Furthermore, the remarkable changes of genes involved in DNA repair possibly indicate the

Sample	LOE Bioaccumulation	LOE Biomarker	WOE
CBZ 14	Severe HQ: 19.0	Moderate HQ: 155.2	MAJOR
VAL 14	Absent HQ: 0	Moderate HQ: 154.2	SLIGHT
CBZ + VAL 14	Moderate HQ: 6.6	Slight HQ: 103.1	SLIGHT
CBZ 28	Absent HQ 0	Moderate HQ: 162.2	SLIGHT
VAL 28	Absent HQ: 0	Slight HQ: 137.0	ABSENT
CBZ + VAL 28	Absent HQ: 0	Slight HQ: 122.1	ABSENT

Fig. 5. Weighted elaboration of whole dataset for each treatment at different.

activation of mechanisms to counteract CBZ-mediated increase of ROS production, consistent with previous investigations in marine invertebrates (Almeida et al., 2021). The lack of significant cellular effects and the limited number of DEGs in co-exposed organisms, provide additional evidence for the role of VAL in reducing mussel's vulnerability to CBZ both at molecular and cellular levels, in agreement with results on bioaccumulation. Worthy to note, the VAL protection of CBZ mediated effects appeared to be weakened at the end of depuration when the number of DEGs increased from 45 to 125 in mixture exposed mussels: it can be hypothesized that a more rapid elimination of VAL compared to CBZ have potentially addressed the delayed onset of observed effects.

Additional insights on effects and mechanism of action of tested drugs are revealed by MN frequency, a typical marker of genotoxicity frequently associated to ROS detrimental effects. In our study a significant increase of MN was observed in organisms exposed to VAL alone, an unexpected result considering that therapeutic effects of VAL include protection from DNA damage in target species, by reducing oxidative stress (Keles et al., 2009). Based on this evidence, the increase of MN in VAL exposed mussels, might be related to changes of cellular turnover, supported at the molecular level by the upregulation of ubiquitin-protein transferase regulatory activity suggesting enhanced protein turnover. At the same time, the lack of MN in CBZ and mixture exposed mussels is consistent with the well-known effect of CBZ as a cell cycle inhibitor (Pérez Martín et al., 2008) confirmed in this study by molecular modulation of several pathways involved in cell cycle regulation, including *CLSPN*, a protein triggering a checkpoint arrest of the cell cycle in response to replicative stress or DNA damage.

The only pathway modulated exclusively by VAL when dosed alone was related to lipid metabolism. The significant induction of Acyl-CoA oxidase, with the activation of β-oxidation of long-chain fatty acids and lipid derivatives, might anticipate possible effects on synthesis of plasmalogens and cholesterol. These results agree with studies on target species, which recognized VAL as regulator of glucose-and lipid metabolism, capable of decreasing levels of cholesterol, possibly due to the link between the angiotensin II receptor type 1-dependent and peroxisome proliferator-activated receptors γ (PPAR γ) signaling pathways (Engeli et al., 2018; Kintscher et al., 2010).

The overall results obtained in this study confirm that an intricate network of mechanisms can regulate responsiveness of non-target marine species to environmental pharmaceuticals, as recently described for different therapeutic classes of pharmaceuticals that potentially interact with both synergistic and antagonistic effects on the same cellular targets and metabolic pathways (Mezzelani and Regoli, 2022). From a broader perspective, a similar scenario, has been recently outlined by an increasing number of studies addressing the toxicity of pharmaceuticals in combination with the multitude of environmental stressors to which organisms are simultaneously exposed, including trace metals and climate-related ocean changes (Almeida et al., 2018a, 2018b; Nardi et al., 2022). In *R. philippinarum* the combined exposures of CBZ with cadmium, or the antihistamine cetrizine with cadmium had lower biological effects than all molecules dosed alone (Almeida et al., 2018a); similarly, the significant changes in mRNA transcription caused by the exposure of *R. philipinarum* to the same pharmaceuticals under ocean acidification conditions (pH 7.5) were characterized by a cross-reactivity with many pathways, triggering unexpected responses (Almeida et al., 2018b).

In the present study, the biological significance of the observed effects was summarized in specific hazard indices allowing an easier qualitative and quantitative comparison of drugs accumulation and biomarkers responses in mussels exposed to different conditions. The Sediqualsoft model applies weighted criteria that consider the toxicological relevance (weight) of measured end points and both the number and magnitude of variations normalized to specific thresholds. In recent years, this approach was validated in several case studies for biological and environmental risk assessment providing the possibility to integrate large dataset of heterogeneous data and to better interpret asynchronous variations of complex pathways (Regoli et al., 2014; Pittura et al., 2018; Mezzelani et al., 2021; Nardi et al., 2022). WoE approach confirmed the greater effects of CBZ alone compared to VAL and the mixture treatments. The risk for organisms exposed to CBZ alone was summarized as MAJOR based on "Severe" accumulation and "Moderate" cellular effects. The SLIGHT risk for mussels treated with VAL and the mixture highlighted the lower vulnerability of these organisms toward VAL and the role of this molecule in reducing the magnitude of adverse outcomes caused by CBZ, both in terms of bioaccumulation and/or cellular effects. Evidence from the present study further allow to characterize immune system and oxidative metabolism as the major pathways contributing to the "Moderate" cellular hazard determined by both CBZ and VAL when dosed alone, and to the antagonistic effect observed after exposure to the mixture.

The significant decrease of the WOE risk level in all treatments at the end of the depuration phase (SLIGHT for CBZ and ABSENT for VAL and CBZ + VAL), provided a quantitative value to the capability of this species to recover detrimental effects caused by pharmaceuticals, highlighting,

however, that cellular effects of CBZ lasted longer (hazard "Moderate") compared to the sharp decrease of bioaccumulation hazard (from "Severe" to "Absent"). Such findings revealed for the first time that combination of CBZ and VAL, in marine species, results in a complex drug-drug interaction, a typical phenomenon for human medicine (Palleria et al., 2013).

In conclusion, the present study revealed a greater biological reactivity of CBZ compared to VAL, providing novel insights on mechanisms of action in non-target species. For the first time clear evidence on antagonistic interactions between these compounds, were related to a reduced effect of the antiepileptic drug on a non-target species. Such outcomes represent a new perspective for a more comprehensive interpretation of pharmaceuticals levels and impacts in wild organisms, further highlighting that the single-chemical approach can significantly lead to both under and -overestimation of the risk, stressing out the importance to move forward integrated approaches, particularly for environmental contaminants of emerging concern.

Statement of environmental implication

Pharmaceuticals are increasingly documented in aquatic environments, posing a serious concern for their toxicity at low concentrations. These contaminants are not included in monitoring guidelines and environmental risk assessment is hampered by limited knowledge on effects in organisms exposed to mixtures.

This study revealed several hazardous responses evoked in marine organisms by a realistic mixture of pharmaceuticals, demonstrating mechanistic interactions and biological reactivity often persisting after the exposure period. The possibility to synthesize huge datasets of heterogeneous data in quantitative hazard indices represent a pivotal procedure for weighted risk assessment, better addressing environmental relevance and ecological consequences of such compounds.

CRediT authorship contribution statement

Mezzelani Marica: Conceptualization, Methodology, Formal analyses, Investigation, Data curation, Writing original draft-Review & editing, Visualization, Project administration; **Luca Peruzza:** Investigation, Formal analyses, Data curation; **Giuseppe d'Errico:** Software; Formal analyses, Data curation; **Massimo Milan:** Investigation, Data curation, Resources, Writing - Review & Editing; **Stefania Gorbi:** Resources, Supervision; **Francesco Regoli**: Conceptualization, Writing - Review & Editing, Supervision, Project administration; Funding acquisition.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors (Marica Mezzelani, Luca Peruzza, Giuseppe d'Errico, Massimo Milan, Stefania Gorbi and Francesco Regoli) 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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Graphical Abstract was created with BioRender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.160465.

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