Venezia2021

Programma di ricerca scientifica per una laguna “regolata”

Linea 2.3

Contaminanti emergenti in laguna, esposizione ed effetti

D2.3.3.3

Caratterizzazione delle risposte nella vongola R. philippinarum a diversi inquinanti emergenti. Analisi chimiche molecolari (trascrittomiche), biochimiche e cellulari in vongole esposte a diversi contaminanti/miscele

D2.3.3.4

Definizione dei meccanismi di azione di diversi inquinanti emergenti e dei possibili rischi per le attività produttive e l’ecosistema

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Sommario

1. Introduzione

Alla luce dell'impatto antropico a cui è soggetta la laguna di Venezia, la valutazione della presenza di contaminanti emergenti assume un ruolo estremamente importante per la salvaguardia dell'ecosistema lagunare e delle attività produttive legate alla molluschicoltura. Oltre a determinare la presenza e la concentrazione di inquinanti in questo ambiente, assume un ruolo cruciale la valutazione dei possibili effetti sulle specie che la abitano. L'integrazione di diverse competenze e tipologie di analisi, dalle analisi molecolari ad analisi biochimiche e cellulari, può permettere di ottenere informazioni sui possibili meccanisimi di azione e quindi sugli effetti dei diversi inquinanti. I nostri studi si sono focalizzati su diverse categorie di inquinanti emergenti presenti nella laguna di Venezia, andando a studiarne, in condizioni controllate, i potenziali effetti in molluschi bivalvi di interesse economico per la laguna di Venezia (vongole e mitili) mediante l'applicazione di metodologie molecolari innovative volte alla caratterizzazione dei profili trascrizionali e delle comunità microbiche residenti negli animali. Queste analisi sono state supportate da ulteriori analisi biochimiche e cellulari, nonchè analisi chimiche di bioaccumulo.

La scelta delle categorie di inquinanti emergenti su cui focalizzare l'attenzione sono state motivate da diversi fattori. Il glifosato e il suo metabolita AMPA rientravano tra gli inquinanti investigati nelle diverse campagna di monitoraggio previste dalla Linea 2.3. Per iniziare quanto prima le attività, l'esposizione in mitili è stata effettuata precedentemente alle diverse campagne di campionamento e analisi, e al momento dell'esposizione non si disponeva di informazioni sulle concentrazioni presenti in laguna di Venezia.

I principali risultati ottenuti da questo studio mostrano la capacità di questi composti di causare perturbazioni al microbiota nella ghiandola digestiva degli organismi oltre che facilitare la diffusione di potenziali patogeni, e inducendo l'attivazione del sistema immunitario dell'organismo ospite, come osservato dalle analisi trascrittomiche. Tutto questo induce a pensare che i contaminanti testati possono esercitare tossicità diretta sui mitili e indiretta causando cambiamenti delle comunità microbiche.

La scelta di analizzare il C6O4, un PFAS di nuova generazione, deriva dalla necessità di acquisire i primi dati sul possibile bioaccumulo e sugli effetti di questo composto in organismi residenti nella laguna di Venezia. Si tratta infatti di un composto del tutto nuovo che, alla luce delle concentrazioni rilevate del delta del Po, sta destando crescenti preoccupazioni. Lo studio ha permesso di determinarne la presenza anche in laguna di Venezia e di ottenere le prime informazioni sui possibili effetti, utilizzando come modello una specie di bivalve di interesse commerciale ed ecologico per la laguna (la vongola filippina *R. philippinarum*).

Ciò che emerge dallo studio è che il C6O4 sembra essere in grado di causare perturbazioni al microbiota della ghiandola digestiva delle vongole, determinando probabilmente la compromissione dell'omeostasi fisiologica dell'ospite inducendo anche modifiche trascrizionali in diverse pathways molecolari. Considerato ciò, l'uso del C6O4 può rappresentare non solo un pericolo ambientale ma anche un potenziale rischio per la salute umana sottolineando così la necessità di ulteriori studi a riguardo.

Infine, mitili sono stati esposti ad amyl salicilato, una delle fragranze maggiormente riscontrate nella laguna di Venezia durante le diverse campagne di monitoraggio. Gli animali sono stati pertanto esposti a concenetrazioni rilevate nel loro ambiente naturale. I dati ottenuti dalle indagini sono in fase di validazione e saranno mostrati tramite la pubblicazione di un articolo scientifico.

Lo studio sugli effetti del glifosato e AMPA è stato pubblicato nel 2020 su Environmental Research (Iori et al., 2020). Le esposizioni a C6O4 e PFOA hanno permesso la pubblicazione di due studi, uno su Environment International, mentre una seconda parte è stata pubblicata su Journal of Hazardous Materials, entrambi nel 2021. Infine lo studio sugli effetti dell'Amyl salicilato è in fase di completamento e verrà verosimilmente sottomesso in una rivista scientifica entro la fine del progetto Venezia2021.

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2. Introduction

Evaluating the presence and the potential effects on organisms of emerging contaminants is important to safeguard the lagoon ecosystem and for the production activities related to shellfish farming. The use of a multidisciplinary approach including molecular, biochemical, cellular and chemical analyses allows to obtain important information regarding the possible mechanisms of action and therefore on the effects of different pollutants. In this context, we performed laboratory experiments to evaluate the bioaccumulation and potential effects of different categories of emerging pollutants detected in the Venice lagoon in bivalve molluscs of economic interest for the Venice lagoon (clams and mussels). To reach such a goal, we applied innovative molecular methodologies (RNA-sequencing and 16S rRNA Amplicon Sequencing) aimed at the characterization of transcriptional profiles and microbial communities accompanied by chemical, biochemical and cellular analyses.

In our experiments, we tested the possible effects on bivalve species of glyphosate and its metabolite AMPA, PFOA and C6O4, and Amyl salicylate. Glyphosate and its metabolite AMPA were investigated in several monitoring campaigns in the Venice lagoon. C6O4, a new generation PFAS, was chosen to fill the lack of information on the bioaccumulation capacity and effects of this compound in farmed clams. Furthermore, to increase solid data we compared results to that of PFOA, a well-studied traditional PFAS over the years by scientific community. Amyl salicylate was tested because it represents one of the main fragrances detected in Venice lagoon during several monitoring campaigns performed within Venezia 2021. The studies here presented were already published in international peer-reviewed journal. In detail, the study on the effects of glyphosate and AMPA on mussels was published in Environmental Research in 2020 (Iori et al. 2020), while two papers reporting results obtained by C6O4/PFOA experiment were published in Environmental International and Journal of Hazardous Materials. Finally, the study on Amyl salicylate is almost complete and it will likely be submitted in a scientific journal by the end of 2021.

3. The effects of glyphosate and AMPA on the Mediterranean mussel *Mytilus galloprovincialis* and its microbiota

3.1 Summary

Glyphosate, the most widely used herbicide worldwide, targets the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway found in plants and some microorganisms. While the potential for glyphosate to induce a broad range of biological effects in exposed organisms has been demonstrated, the global molecular mechanisms of toxicity and potential effects in bacterial symbionts remain unclear, in particular for ecologically important marine species such as bivalve molluscs. Here, the effects of glyphosate (GLY), its degradation product aminomethylphosphonic acid (AMPA), and a mixture of both (MIX) on the mussel *M. galloprovincialis* were assessed in a controlled experiment. For the first time, next generation sequencing (RNA-seq and 16S rRNA amplicon sequencing) was used to evaluate such effects at the molecular level in both the host and its respective microbiota. The results suggest that the variable capacity of bacterial species to proliferate in the presence of these compounds and the impairment of host physiological homeostasis due to AMPA and GLY toxicity may cause significant perturbations to the digestive gland microbiota, as well as elicit the spread of potential opportunistic pathogens such as *Vibrio spp.*. The consequent host-immune system activation identified at the molecular and cellular level could be aimed at controlling changes occurring in the composition of symbiotic microbial communities. Overall, our data raise further concerns about the potential adverse effects of glyphosate and AMPA in marine species, suggesting that both the effects of direct toxicity and the ensuing changes occurring in the host-microbial community must be taken into consideration to determine the overall ecotoxicological hazard of these compounds.

3.2 Introduction

The glyphosate [N-(phosphonomethyl) glycine] is a nonselective, systemic herbicide, ranked among the most extensively used agricultural chemicals worldwide (Annett *et al.*, 2014; Cattani *et al.*, 2014; Myers *et al.*, 2016; Van Bruggen *et al.*, 2018, Green *et al.*, 2018). This organophosphorus compound is able to inhibit the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway that participates in the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan in bacteria, fungi, and plants (Schönbrunn *et al.*, 2001; Shehata *et al.*, 2013; Shilo *et al.*, 2016). Glyphosate is considered one of the least toxic pesticides used in agriculture on the basis of its low contamination potential in surface and/or groundwater and its inability to affect animals, which lack the shikimate pathway (Vereecken 2005, Duken *et al.* 2008). However, due to its widespread use in agriculture, forestry, urban areas, and aquaculture as a plant growth regulator, glyphosate can easily spread throughout ecosystems, including surface waters, thus reaching plants, animals, and the food chain. Recently, several studies on glyphosate and amino-methylphosphonic acid (AMPA) - the product of the microbial degradation of glyphosate- have highlighted a wide range of toxicological effects in non-target organisms, raising concerns about the potential risks to the aquatic environment (Struger *et al.*, 2008. Battaglin *et al.*, 2009; Wang *et al.*, 2016, Skeff *et al.*, 2015; Mercurio *et al.* 2014). For instance, effects on energy metabolism and reproduction, increased oxidative stress, inhibition of acetylcholinesterase (AChE), genotoxicity and disruption of pro-apoptotic signalling have been described in fish following glyphosate exposure (Cavalcante *et al.*, 2010, Modesto and Martinez, 2010; Guilherme *et al.*, 2010, 2012; De Castilhos Ghisi and Cestari, 2013; Uren Webster *et al.*, 2014, Uren Webster and Santos, 2015). Similarly, recent studies have demonstrated that environmentally realistic concentrations of glyphosate and AMPA can affect haemocyte parameters in bivalve species (Matozzo *et al.*, 2018a; Matozzo et al,. 2018b; Matozzo *et al.*, 2019a; Matozzo *et al.*, 2019b), and lead to the disruption of

genes involved in apoptosis, immune response, energy metabolism, Ca^{2+} homeostasis, cell signalling and endoplasmic reticulum stress response (Milan *et al.*, 2018a). While glyphosate is known to affect the soil and aquatic microbial communities through the inhibition of the shikimate pathway, its effects on the animal-resident microbiota is beginning to draw increasing attention, in particular regarding the gut microbiota. Glyphosate residues have been shown to perturb the gut microbiota of mammals (mice, rat and poultry), reptilians, and honey bees, highlighting variable susceptibility of microbial species to glyphosate. (Shehata *et al.*, 2013; Lozano *et al.*, 2017; Kittle *et al.*, 2018; Aitbali *et al.*, 2018; Dai *et al.*, 2018; Blot *et al.*, 2019). In particular, exposure of honey bees to glyphosate led to the perturbation of their beneficial gut microbiota, affecting bee health and their effectiveness as pollinators (Motta *et al.*, 2018; Blot *et al.*, 2019). Overall, these studies have demonstrated that the disruption of the host-microbiota relationships following glyphosate exposures may contribute to the onset of pathological conditions. This is of particular relevance in aquatic species in which the spread of opportunistic pathogens following multiple biotic and/or abiotic stresses is ever more frequently suggested to play key roles in extended mortality events (Coma *et al.*, 2009; Vezzulli *et al.* 2010; Vezzulli *et al.*, 2013; Vezzulli *et al.*, 2015; Milan *et al.*, 2019). Here, we seek to decipher for the first time the complex host-microbiota interactions following glyphosate and AMPA exposures in a marine species, focusing on the Mediterranean mussel *M. galloprovincialis*, a widespread bivalve species of commercial interest in marine coastal areas that is extensively used as model organism in ecotoxicological studies. Our study uses a broad and innovative approach based on the combination of RNA-sequencing and 16S microbiota analyses to investigate both short- and long-term exposure (7 and 21 days) to environmentally realistic concentrations of glyphosate (100 µg/L; GLY), AMPA (100 µg/L) and a mixture of the two compounds (100 + 100 µg/L; MIX). Additionally, gene expression analyses provided a wide overview on the molecular mechanisms of action of GLY and AMPA in this filter-feeding species.

3.3 Material and methods

The samples analysed in this study were collected during an experiment already reported by Matozzo *et al.* (2019b). Briefly, specimens of *M. galloprovincialis* (about 5-6 cm shell length) were sampled in a licensed area for bivalve culture in the Lagoon of Venice (Italy). Before the experiments, bivalves were acclimated in the laboratory for one week in large aquaria with aerated seawater (salinity of 35 ± 1, temperature of 18 ± 0.5 °C) and were fed daily *ad libitum* with microalgae (*Isochrysis galbana*). In order to implement the experimental treatments, two independent stock solutions (0.1 g/L, in distilled water) of glyphosate (GLY) and AMPA (Sigma-Aldrich, Milano, Italy) were prepared, and exposure concentrations were obtained by diluting the stock solution in seawater. Bivalves (n = 70 per experimental condition) were exposed for 7 and 21 days to 0 (control; CTRL), 100 µg/L of glyphosate (GLY), 100 µg/L of AMPA and a mixture of 100 µg/L of glyphosate + 100 µg/L of AMPA (MIX). Such concentrations were selected on the basis of information on both glyphosate and AMPA levels in aquatic ecosystems (Skeff *et al.*, 2015; Wang *et al.*, 2016). Throughout the exposure, stock and working solutions, seawater and microalgae were renewed every two days. Analytical verification of exposure concentrations tested in this study were performed in the context of previous studies (Matozzo *et al.,* 2018a; Matozzo *et al.,* 2018b). Following exposure, 20 mussels per treatment were opened, sex was determined by gamete identification under a light microscope, and digestive glands were excised and frozen in liquid nitrogen and stored at -20°C for gene expression and microbiota analyses. Total RNA was extracted from 6 individuals for each condition using RNeasy Mini Kit (Qiagen, Hilden, Germany). The same extracted RNA from each sample was considered for both gene expression (RNA-Seq) and microbiota analyses (16S). The cDNA libraries for RNA-seq analysis were constructed using a Sure Select Strand-Specific mRNA Library (Agilent) according to the manufacturer's protocol (see Milan *et al.,* 2018). The library pools were sequenced on 4 lanes of a HighSeq 4000 (Illumina, Davis, CA, USA) with a single 1*100 bp setup using 'Version2' chemistry, obtaining a total of 1,108,813,603 reads (sequences available in NCBI SRA;

https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA561723). For microbiota analyses, 1 µg of RNA was retro-transcribed to cDNA using the Superscript IV kit (Invitrogen, Life Technologies, Monza, Italy). cDNA was diluted to 0.2 ng/µL and amplified in a 50 µL reaction including 5 µl diluted DNA and 1.5 µL of both reverse and forward primers (10 µM) that specifically target the V3-V4 gene region of the bacterial 16S rRNA as described by Milan *et al.* (2018b). Libraries were then pooled together based on their concentrations and the final pool was quantified using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, United States) and sequenced by BMR Genomics (Padova, Italy) with a Miseq Illumina 2x300. The microbiome sequencing generated 12,132,960 reads, averaging about 252.000 reads per sample (sequences available in NCBI Sequence Read Archive SRA https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA562940). For gene expression analyses after the initial quality check of the raw sequencing data (FastQC report), adapter trimming was carried out using Trimmomatic (Bolger *et al.*, 2014) with the following parameters: headcrop 5, Illuminaclip removed, sliding window 5:20 and minimum length 70. Trimmed RNA-Seq reads of each sample were mapped on the reference *Mytilus galloprovincialis* transcriptome (Moreira *et al.,* 2015). Mapping on reference transcriptome was carried out with Burrows-Wheeler Alignment Tool (Li *et al.,* 2009). Bwa mem was employed using default parameters (mismatch penalty 4, gap open penalty 6, gap extension penalty 1) and allowing a unique match. Reads sorting and count were performed on PICARD and Samtools, respectively. 79.03% of the total reads were successfully mapped, with a mean value of 19.5 million reads per sample. Gene-expression data were then analysed with Multi Experiment View-cluster software (Howe *et al.*, 2011). After TMM Normalization, pairwise comparisons at each time point were performed using EdgeR (p-value < 0.05; Fold Change (FC) >2) (Robinson *et al.*, 2009). For microbiota analyses, Raw reads were trimmed, merged and analysed with QIIME 2 (Quantitative insights into microbial ecology 2; Bolyen *et al.*, 2019) using DADA 2 (Callahan *et al.*, 2016) to obtain high quality representative sequences (named features) before the alignment through MAFFT software (Katoh *et al.,* 2013). Reads were trimmed based on the sequence of primers and the quality of nucleotide assignment. Reads with quality lower than 20 (Phred Score) were discarded. After the quality-filter step, removal of chimeric fragments and reads merging, a total of 3,198,227 reads were obtained. Sequences were classified by QIIME 2 using Python library scikit-learn. Taxa assignment was carried out using SILVA-trained database (Yilmaz *et al.*, 2014). All samples were then rarefied to 38,364 reads. Rarefaction was performed using the package phyloseq (v1.26.1) with the command rarefy_even_depth in R (v3.5.3). From the rarefied Amplicon Sequence Variants (ASVs) table, the observed species richness was calculated. The effects of the experimental treatments, time point, and their interaction on alpha diversity were assessed based on a two-way ANOVA using the function aov in the package stats (v3.6.1). When "treatment" and/or "time" or their interactions were significant, pairwise comparisons among group levels were performed based on Tukey's Honest Significant Differences (Tukey HSD). In order to assess the differential abundance of ASVs across treatments within a single time period, we used the R package DESeq2 (v1.22.2) (Love *et al.*, 2014) on a pre-filtered and unrarefied ASV table. DESeq2 is based on negative binomial generalized linear models and includes a Wald post-hoc test for significance testing. P-values were adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) accounting for multiple comparisons. In order to define the total bacteria and *Vibrio* community load, DNA extraction was performed using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) on the digestive gland of each mussel employed for gene expression analyses and microbiota characterization. After extraction, the quality of DNA was assessed by agarose gel electrophoresis 1%. Quantification of total 16S rDNA and specific *Vibrio* genus 16S rDNA genes were performed using quantitative PCR (qPCR). All amplification reactions were analyzed using a Roche LightCycler 480 Real-Time thermocycler (Roche Applied Science, Penzberg, Germany). The total qPCR reaction volume was 10 µl and contained 2.5 µl DNA (1.5 - 9 ng/µl) and 7.5 µl LightCycler 480 PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, Massachusetts, U.S.) with 10 µM PCR primer (Eurofins, Bruxelles). Total bacteria specific primer pairs were the 16S_Fw – TCCTACGGGAGGCAGCAGT and 16S_Rev-GGACTACCAGGGTATCTAATCCTGTT, targeting the variable V3-V4 loops for bacterial communities (Nadkarni *et al.*, 2002). Total *Vibrio* specific primer pairs were Vib1-Fw GGCGTAAAGCGCATGCAGGT and Vib2-Rev

GAAATTCTACCCCCCTCTACAG (Siboni *et al.*, 2016), targeting the 16S rRNA of *Vibrio* genus. As a reference gene we considered the universal 18S rRNA gene, using the specific primer pairs bivalve_uni_Fw CCGATAACGAACGAGACTC and bivalve_uni_Rev CACAGACCTGTTATTGCTC.

3.4 Results

3.4.1 Mussel gene expression analyses

Transcriptional changes in response to GLY, AMPA and their mixture (MIX), were assessed through pairwise comparisons between the control and treated mussels at 7 and 21 days of exposure. At 7 days, the majority of differentially expressed genes (DEGs) was found in the response to AMPA exposure (121 DEGs). At 21 days, the response to AMPA diminished (78 DEGs), while an opposite trend was observed in GLY- and MIX-exposed mussels, showing a total of 129 and 140 DEGs, respectively (Table 1). The complete lists of DEGs are summarized in Table 2.

Table 1. Number of differentially expressed genes obtained for each comparison. CTRL: control; GLY: glyphosate; MIX: mixture (AMPA+GLY).

Comparison	N° Differentially expressed genes	N° Up-regulated genes	N° Down-regulated genes
CTRL vs AMPA 7 days	121	59	62
CTRL vs GLY 7 days	78	32	46
CTRL vs MIX 7 days	76	34	42
CTRL vs AMPA 21 days	78	49	29
CTRL vs GLY 21 days	129	45	84
CTRL vs MIX 21 days	140	53	87

Table 2. Summary of differentially expressed genes identified in response to AMPA, glyphosate (GLY) and GLY+AMPA (MIX). Up- and down-regulated genes in treated mussels are reported in red and green, respectively. Numbers in parentheses represent the number of significant contigs for the reference gene (Full list of differentially expressed genes are reported in Iori et al. 2020; https://doi.org/10.1016/j.envres.2019.108984).

PATHWAYS	AMPA 7 DAYs	AMPA 21 DAYs	GLY 7 DAYs	GLY 21 DAYs	MIX 7 DAYs	MIX 21 DAYs
Xenobiotic metabolism and detoxification	SLC22A13, MGST1, MRP5, MRP9, HIP(3)	MGST1, HIP (2)	MGST1, HIP	HIP (2)	MRP5, SLC22A13, HIP	
Apoptosis regulation	GIMAP4 (1), GIMAP4 (6), GIMAP7 (2), GIMAP8 (2), PARP14, I27L1, IFI27L2B, IAP1, BIRC1, CASP8	GIMAP7, IFI6, I27L1	GIMAP4 (5), GIMAP 7 (1). GIMAP4 (2) , GIMAP7 (1), GIMAP8 (1), BIRC1, PARP14,	IFI6, I27L1, IFI27L2B, GIMAP4, GIMAP5, IAP2, BIRC3, IAP1	GIMAP4 (7), GIMAP7 (2), GIMAP8 (2), GIMAP4 (1), GIMAP8 (1), PARP14, I27L1	GIMAP7 (3), IFI6, I27L1, CASP2

PATHWAYS	AMPA 7 DAYs	AMPA 21 DAYs	GLY 7 DAYs	GLY 21 DAYs	MIX 7 DAYs	MIX 21 DAYs
Oxidative stress response		SOD		SOD		SOD
Neurotransmitter / synaptyc transmission	EAA1, PCLO	CHRNA9, CBLN1, ADGRB3	EAA1, PCLO, CHRNA2	CHRNA9, CHRNA2, PCLO, CBLN1		CHRNA9, CHRNA2, ADGRB3, PCLO
Immunological processes and inflammation	I27L1, C1QTNF3, C1QTNF6, C1QTNF9, IIGP5, IFI27L2B, MYNB, FCN1 C1QTNF4 C1QB, COL12	LITAF, IFI44, IFI6, I27L1, C1ql2, FCN2, C1QB, C1QTNF4,	MGD1, C1ql3	LITAF, IIGP5, IFI6, C1ql2, I27L1, MYNB, IFI27L2B, C1QTNF6, TLR2 C1QTNF4, C1QTNF5, BDEF, COL12	I27L1, FCN2	LITAF, , IFI6, C1ql2, I27L1, C1QTNF6, C1QB, BDEF
Glycogen metabolism				PYG, PYGB, PYGM		PYG, PYGB,
Cell Cycle	CDN1C, CENPE					
Cellular response to stress		HSP12A	HSP12B, HSP12A	HSP12A, HSP12B		HSP12A, HSP12B

Venn diagrams were then constructed to represent the significant transcripts that are differentially regulated in common across different treatments and time points (see Figure 1). AMPA and GLY showed specific transcriptomic response, with only 11.5% of DEGs found in common between the two treatments. Conversely, results for MIX exposure suggests a cumulative effect of AMPA and GLY exposures, with the 46% of significant transcripts found differentially expressed also in response to AMPA and/or GLY exposures (Figure 1).

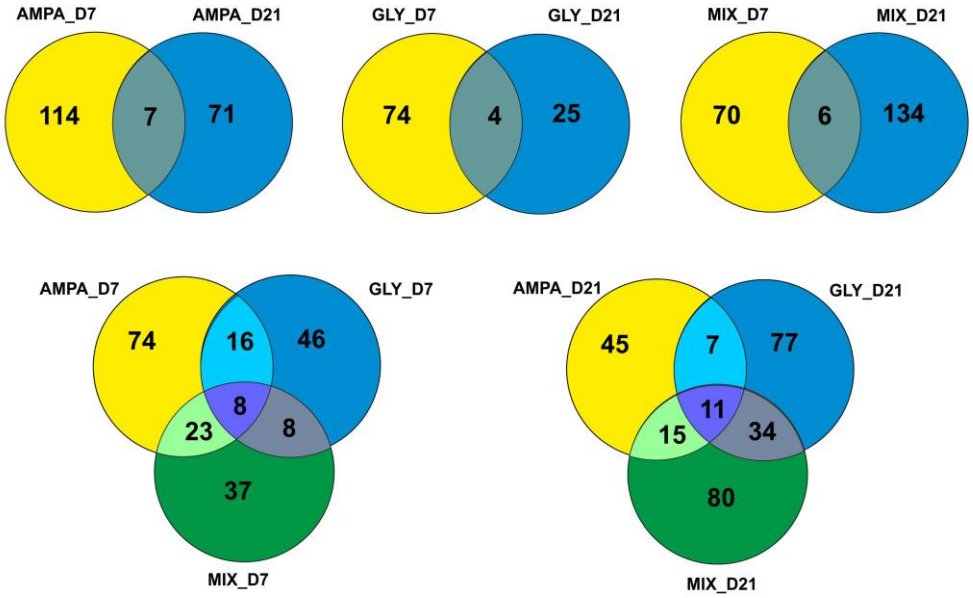

Figure 1. Venn diagrams reporting the number of differentially expressed genes for each pairwise comparison among control and exposed groups.

Among the eight transcripts commonly found differentially expressed among treatments at 7 days, a putative *Poly [ADP-ribose] polymerase 14 (PARP14) and* three transcripts coding for *GTPase IMAP family member 4 (GIMAP4)* should be highlighted. The GIMAP family, an expanded gene family in oysters (McDowell *et al.*, 2016), plays a role in defense cell differentiation and apoptosis (Carter *et al.,* 2007, McDowell *et al.*, 2014, Heinonen *et al.,* 2015). Noteworthy, in addition to GIMAP4, several contigs representing putative GIMAP5, GIMAP7 and GIMAP8 were also found to be responsive to GLY, AMPA, and MIX, in particular after 7 days of chemical exposures (Table 2). *PARP14* is a member of the PARP protein family and codes for an anti-apoptotic protein that is involved in cell stress responses (Vyas *et al.*, 2014). Among the commonly up-regulated genes at 7 days, *Heavy metal-binding protein* (HIP) showed a fold change higher than 3.4 in all treatments.

After 21 days, 11 genes were found up-regulated in common across all treatments (GLY, AMPA, and MIX). Among them, *superoxide dismutase* (*SOD*), *Heat shock 70 kDa protein 12A* (HSP12A), and putative *Neuronal acetylcholine receptor subunit alpha-9* (*CHRNA9*) always showed a substantial fold change (FC>3). Up-regulation in all treatments was observed for four genes putatively involved in immunological processes, inflammation, and regulation of apoptosis, such as *Lipopolysaccharide-induced tumor necrosis factor-alpha factor-like protein* (*LITAF*), an important transcription factor that mediates the expression of inflammatory cytokines, *Interferon alpha-inducible protein 6* (*IFI6*), *Interferon alpha-inducible protein 27-like protein 1* (*I27L1*), and *Complement C1q-like protein 2* (*C1ql2*).

AMPA exposure altered the expression of several additional genes potentially involved in immune response at both time-points, such as several C1q domain-containing (C1qDC) proteins, *Interferon-inducible GTPase 5* (*IIGP5*; up-regulated at 7 days), *Interferon alpha-inducible protein 27-like protein 2B* (*IFI27L2B*; up-regulated at 7 days), *interferon induced protein 44 like* (*IFI44*; up-regulated at 21 days; McDowell *et al.,* 2016), *Myticin-B (MYNB;* up-regulated at 7 days), *ficolin-1* (*FCN1*) and *ficolin-2* (*FCN2*). Up-regulation of genes involved in xenobiotic metabolism, such as *Microsomal glutathione S-transferase 1* (*MGST1*) and *Multidrug resistance-associated proteins* (*MRP5*, *MRP9*), were also found following AMPA exposure. Transcriptomic profiles of AMPA-exposed mussels also suggests the disruption of genes involved in apoptosis regulation (e.g. *Apoptosis 1 inhibitor* at 7 days, *caspase-8* at 21 days), regulation of cell proliferation and cell cycle (*Cyclin-dependent*

kinase inhibitor 1C, CDN1C; *Centromere-associated protein E, CENPE*), and neurotransmission (*Excitatory amino acid transporter 1, EAA1; Protein piccolo, PCLO; CHRNA9*).

Similarly, mussels exposed to GLY showed the up-regulation of *HIP* at 7 and 21 days and of *MGST1* at 7 days (FC>10), as well as altered expression of several genes involved in immune response and regulation of apoptosis at both time-points. Among them, *defensin MGD-1* was up-regulated at 7 days, while *IFI6*, *Toll-like receptor 2* (*TLR2*), *MYNB*, *I27L1*, *IFI27L2B*, and *IIGP5* were up-regulated after 21 days of GLY exposure. Among genes involved in apoptosis regulation, *Baculoviral IAP repeat-containing protein 1* (*BIRC1)*, *Apoptosis 2 inhibitor* (*IAP2),* and *BIRC3* were found up-regulated at both time-point. It should also be noted that several genes involved in synaptic transmission and neurotransmission, such as *Excitatory amino acid transporter 1* (*EAA1*), *Neuronal acetylcholine receptor subunit alpha-2* (*CHRNA2*), *Protein piccolo (PCLO)*, *CHRNA9* and *Cerebellin-1* (*CBLN1*), were found differentially expressed in response to GLY, similar to the results obtained in mussels exposed to AMPA.

After 7 days, MIX exposed mussels showed differential regulation of several genes involved in cell adhesion (*aggregan, ACAN*; *collagen type VIII alpha 1 chain*, *COL8A1*; *collagen type XII alpha 1 chain*, *COL12A1*; *myosin-10*, *MYH10*; *myosin-4*, *MYO4*; *myosin-11*, *MYH11*; *Myosin heavy chain*, *MISS*), transmembrane transport **(***MRP5; Solute carrier family 22 member 13, S22AD),* and xenobiotic metabolism and detoxification (*MRP5, SLC22A13*). Several putative GIMAP family members (13 genes), genes involved in immune response (*IFI6; C1qDC proteins; I27L1; BDEF*), and *HIP* showed profiles similar to those detected in response to single GLY and AMPA exposure. Disruption of genes involved in cell adhesion was confirmed after 21 days of exposure to MIX (*titin*, TTN; *elastin microfibril interfacer 1, EMILIN1; elastin microfibril interfacer 2, EMILIN2; semaphorin 5A, SEM5A*). At this time-point, genes involved in apoptotic process (*caspase 2*, *LITAF*), and neurotransmission and synapses regulation (*adhesion G protein-coupled receptor B3, ADGRB3; CBLN1; CHRNA9, CHRNA2*, *PCLO*) showed profiles similar to those detected in response to single AMPA and GLY exposure. To conclude, several *Glycogen phosphorylase* (*PYG, PYGB, PYGM*) were commonly down-regulated in mussels exposed to GLY and MIX for 21 days.

3.4.2 Mussel digestive gland microbiome

Mussel-associated bacterial communities consisted primarily of Gammaproteobacteria (38–62%), Bacteroidia (10.8–21.4%), Alphaproteobacteria (5.9–23.6%), Mollicutes (1.6–16.3%), Fusobacteriia (0.3–12%), Deltaproteobactria (0.3-5.1%), and Spirochaetia (0.5-6.6%). Patterns of alpha diversity showed a significant effect of “treatment” on microbial species richness (ANOVA, p=0.04; data not shown). In particular, a trend of lower microbial species richness was detected in MIX-exposed mussels compared to CTRL after 21 days (p-value>0.05; Figure 2A).

Mussels exposed to GLY significantly differed in the abundance of 17 and 19 ASVs after 7 and 21 days respectively, compared to CTRL. Among them, 8 and 10 ASVs were present in greater abundance in GLY after 7 and 21 days, compared to CTRL (log2 FC 5.8 to 23.2), including five members from the genus *Vibrio* at 7 days. After 21 days, in mussels exposed to GLY ten ASVs were significantly more abundant (log2 FC 6.6 to 22.4). This included members from the families Vibrionaceae (genus *Vibrio*), Kangiellaceae (genus *Aliikangiella*), Pseudomonadaceae, Alteromonadaceae, Nitrincolaceae, Cellvibrionaceae and Saccharospirillaceae (genus *Oleispira*).

Mussel exposure to AMPA led to smaller changes in microbiome composition, with a total of 6 and 10 differentially represented ASVs at 7 and 21 days, respectively. Specifically, 2 and 8 ASVs were present at greater abundance in AMPA at 7 and 21 days respectively, compared to CTRL. Among them, one sequence from the genus *Aliikangiella* and one member of the family Flavobacteriaceae were identified at 7 days (log2 FC 7.8 and 22.2). At 21 days, 2 members from the families Vibrionaceae (genus *Vibrio*) were identified as well

as taxa from the families Rhodobacteraceae, Cellvibrionaceae, Saprospiraceae (genus *Aureispira*) and Sandaracinaceae (log2 FC 7.4 to 22.7).

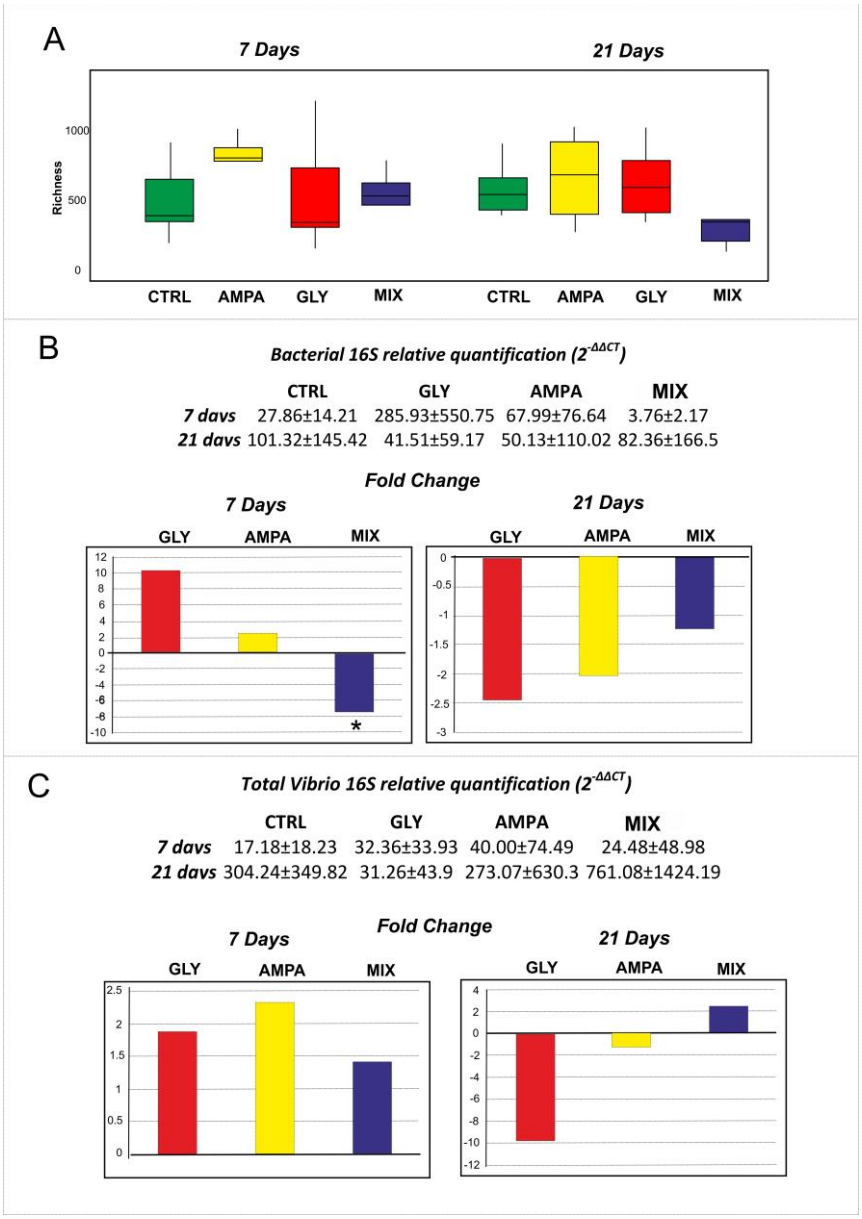

Figure 2. (A) Richness diversity index for each group at 7 and 21 days. CTRL: control group; GLY: glyphosate exposure; MIX: AMPA+GLY; (B and C) Relative quantification of total bacteria (B) and total Vibrio load (C) obtained by qPCR. qPCR was performed in the same individuals considered for gene expression and microbiota analyses. The 2-ΔΔCq reported in table 2B and 2C represent the mean value and the standard deviation of the 6 samples analyzed for each group. The fold change reported in 2B and 2C charts has been obtained calculating ratio between the 2-ΔΔCq of each treated group (GLY, AMPA, MIX) and MIX) and CTRL group. * indicate statistical significance (p-value<0.05; Mann-Whitney test).

Significant changes in digestive gland microbiota composition was also found in mussels exposed to MIX, with a total of 15 and 12 ASVs that significantly differed in abundance at 7 and 21 days, respectively. However, at variance with what observed in mussels exposed to GLY and AMPA, the majority of significant ASVs were

down-represented in MIX-exposed mussels (22 out of 27 significant ASVs). Among the differentially represented taxa, *Oceanospirillales* (4 ASVs) and *Flavobacteriales* (5 ASVs) should be mentioned, while no significant OTUs representing *Vibrio spp*. were found.

3.4.3 qPCR for total bacteria and Vibrio quantification

To determine the effects of GLY, AMPA and MIX on the size of the gut microbiome, relative abundances of digestive gland bacteria were assessed through quantitative PCR (qPCR) applied to total 16S rRNA. Considering that the *Vibrio* genus include species pathogenic to bivalves that are known to be associated with abnormal mortality outbreaks in farmed animals (e.g. King *et al.*, 2012; Petton *et al.*, 2015; Le Roux 2016), total *Vibrio* load was also investigated. Likely due to the high inter-individual variability (Figure 2B and 2C), no significant difference in total bacterial load was observed when comparing AMPA and GLY exposures to the CTRL group ($p>0.05$, Mann-Whitney test; data not shown). A significant decrease of total bacterial load was observed only in MIX exposures at 7 days ($p\text{-value}<0.05$; Mann-Whitney test; Figure 2B), possibly due to a cumulative effect of GLY and AMPA. Similarly to total bacteria load, total *Vibrio* load showed high inter-individual variability and no significant difference was observed when comparing AMPA, GLY, and MIX exposures to the CTRL group ($p>0.05$, Mann-Whitney test). However, we can note a slight increase in all treatments at 7 days compared to the CTRL group, and a decrease of total *Vibrio* load in GLY-exposed mussels at 21 days compared to the CTRL group (Figure 2C).

3.5 Discussion

In a previous study, the effects of GLY, AMPA, and a mixture of both were assessed in haemocytes using biochemical parameters (Matozzo *et al.*, 2019b). The present study, which is a continuation of the previous one, investigated for the first time gene expression and microbiome profiles of mussels following exposure to three different treatments (GLY, AMPA, and MIX), providing unprecedented information regarding the molecular mechanisms of action of these compounds in both the host and its microbiota.

3.5.1 Glyphosate and AMPA perturb the digestive gland bacterial community

Considering that GLY may affect bacterial symbionts through the inhibition of EPSPS, our previous study suggested that the identified transcriptional changes observed in genes involved in the immune response, energy metabolism, and digestion following GLY exposure may be in part linked to changes occurring in host-microbiota composition (Milan *et al.*, 2018a).

To date, fluctuations in the host microbiota following glyphosate exposure have been demonstrated in only a few terrestrial species (Kittle *et al.*, 2018; Shehata *et al.*, 2013; Aitbali *et al.*, 2018; Lozano *et al.*, 2017; Dai *et al.*, 2018), while dynamics of microbial communities following GLY exposure have never been described in marine species. Here, Next Generation Sequencing (NGS) analysis of 16S rRNA sequences provided an in-depth characterization of the Mediterranean mussel microbiota across controlled exposures to GLY, AMPA, and a mixture of both. These analyses have been complemented by the quantification of total bacteria and total *Vibrio* community load through qPCR.

To our knowledge, only one study has so far investigated changes occurring in host-microbiota in response to AMPA. While a reduction in growth of specific bacterial species was demonstrated *in vitro*, AMPA did not appear to induce significant changes in the honeybee microbiota (Blot *et al.*, 2019). In our study, AMPA exposure led to slight changes in the microbial community of mussels, while substantial modifications were

detected in mussels exposed to GLY and MIX at both time-points. The considerable changes in the MIX group suggest a possible cumulative effect of AMPA and GLY exposures (100 + 100 µg/L) that may have led to the rapid and significant reduction of the total bacteria load detected at 7 days. This hypothesis is supported by the majority of significant ASVs found down-represented in MIX exposed mussels compared to CTRL (22 out of 27 significant ASVs).

The *Vibrio* genus, which was over-represented in GLY (5 taxa at 7 days) and AMPA (2 taxa at 21 days) exposed mussels, was described to be among the dominant fractions of *M. galloprovincialis* microbiota (Vezzulli *et al.*, 2018). In our study, the increased dominance of bacteria belonging to this genus at 7 days in all treatments, while not significant (Figure 2C), may be related to possible new environmental niches that are made available through a re-organization of the microbiota following GLY and AMPA exposures. Such an event is in line with the opportunistic nature of *Vibrio* spp. that may spread following stressful environmental conditions, as widely observed in several marine invertebrate species (e.g. Frydenborg *et al.*, 2013; Vezzulli *et al.,* 2015; Milan *et al.,* 2019).

On one hand, the changes described in microbiota composition can be linked to the antimicrobial activities of both GLY and AMPA that may differentially affect symbionts of the digestive gland. On the other hand, a compromised physiological status due to the toxicity of AMPA and GLY, as suggested by gene expression profiling (see below), could also facilitate the spread of opportunistic pathogens. While the direct effects of AMPA and GLY on the mussel microbiota should be clarified in future studies through the *in vitro* isolation of digestive gland-associated bacteria, the characterization of host-gene expression profiles has identified their potential molecular mechanisms of action and established possible host responses to changes in microbiota composition.

3.5.2 Host-immune system activation may control microbiota changes following glyphosate and AMPA exposure

Apoptosis-associated and IAP proteins, found differentially expressed in response to both GLY and AMPA exposures (e.g. *IAP1, IAP2, BIRC1, BIRC3, PARP14*), may play key roles in the defense response of bivalves to pathogens (Sunila *et al.*, 2003; Donaghi *et al.*, 2009; Sokolova *et al.*, 2009; Hughes *et al.*, 2010; Brulle *et al.*, 2012; McDowell *et al.*, 2014; Rey-Campos *et al.*, 2019). Among the main modifications detected at the molecular level in all three treatments, several members of the GIMAP family, representing an expanded gene family in bivalve species (McDowell *et al.*, 2016; Zhang *et al.,* 2012), were confirmed to be a potential target during GLY exposure, as proposed in our previous study (Milan *et al.*, 2018a). Recently, McDowell and colleagues (2014 and 2016) found a high and unprecedented diversity of GIMAP transcripts to be mostly down-regulated following bacterial challenge in the eastern oyster *C. virginica*. The authors suggested that GIMAPs may promote the survival of haemocytes by negatively regulating apoptosis, as previously proposed in the case of human monocytes (Dower *et al.*, 2008). In our study, a total of 10, 6, and 11 members of the GIMAP family, represented by more than 15 significant contigs, were down-regulated at 7 days in response to AMPA, GLY, and MIX, respectively (Table 2). The over-representation of *Vibrio spp.* at this time-point supports the transcriptional changes observed in other studies, where down-regulation of GIMAP family members has been reported after bacterial challenges including *Vibrio* infections (McDowell *et al.*, 2014; Rey-Campos *et al.*, 2019).

Several genes involved in immune response and inflammation were also transversely found differentially expressed in all treatments (Table 2). Among them, *LITAF* was over 3 times up-regulated in all treatments after 21 days. *LITAF* is an important transcription factor that mediates the expression of inflammatory cytokines, including TNF-α, in lipopolysaccharide (LPS)-induced processes. Recently, *LITAF* has been proposed to be a potent factor in the regulation of genes that are involved in innate immune defense mechanisms in

bivalves. In particular, the up-regulation of *LITAF* was demonstrated in the oyster *C. gigas* challenged subjected to a mixture of pathogenic *Vibrio* species (Park *et al.*, 2008), as well as in cultured haemocytes activated with LPS (Yu *et al.,* 2007).

Superoxide dismutase (*SOD*) was also up-regulated in response to all treatments after 21 days. SOD, an antioxidant enzyme widely used as biomarker of oxidative stress in ecotoxicology, may also contribute to innate immunity by preventing the accumulation of Reactive Oxygen Species (ROS), potent immune effectors with antimicrobial properties that can be rapidly generated following biotic challenges such as *Vibrio* infections (Labreuche *et al.*, 2006; Buggè *et al.*, 2007; Le Bris *et al.*, 2015). An overall increase in the transcription of antioxidant genes following *Vibrio* infection was also described in the digestive gland of *M. galloprovincialis*, suggesting that this species can mount an efficient antioxidant response towards *Vibrio* species (Canesi *et al.*, 2010).

To conclude, within the list of genes involved in immune response and inflammation reported in Table 2, the up-regulation of *Myticin-B* (*MYNB*), *Ficolins* (FCN-1, FCN2) and *Defensin MGD1,* also suggested the activation of an immune response following the spread of pathogenic opportunists (Romero *et al.*, 2011; Xiang *et al.*, 2014; Moreira *et al.*, 2018a; Moreira *et al.*, 2018b). Furthermore, this hypothesis is strongly supported by the increased haemocyte proliferation highlighted in Mediterranean mussels following AMPA and/or GLY exposures (Matozzo *et al.*, 2018a; Matozzo *et al.,* 2018b; Matozzo *et al.,* 2019b).

3.5.3 Molecular changes following glyphosate and AMPA exposures suggest alterations of M. galloprovincialis physiological homeostasis

Overall, RNA-seq analyses demonstrated a transient and time-dependent transcriptional response to each treatment. Similarly, the Venn diagrams suggested that the majority of transcriptional changes were treatment-specific to AMPA and GLY treatments, while in MIX-exposed mussels a cumulative effect appears to occur. However, most of the disrupted molecular pathways suggested similar mechanisms of toxicity for AMPA and GLY.

Noteworthy, as reported in our previous study (Milan *et al.*, 2018), possible effects on neurotransmitter transporter activity and synaptic transmission have been highlighted in response to both AMPA and GLY exposures. Among the most interesting genes, *CHRNA9*, *CHRNA2*, *PCLO* and *EAA1* deserve particular attention. *PCLO,* found differentially expressed also in our previous study in response to GLY (Milan *et al.*, 2018), plays a role in synaptic vesicle trafficking and in the organization of synaptic active zones. *EAA1,* also known as *Solute Carrier Family 1 Member 3* (*SLC1A3*), functions in the termination of excitatory neurotransmission in the central nervous system by removing the released glutamate from the synaptic cleft. Similarly, disruption of *Sodium-and chloride-dependent glycine transporter 1 (SLC6A9)*, a transporter that limits the signaling of glycine, was also observed in response to GLY in our previous studies (Milan *et al.*, 2018). Given glyphosate's structural similarity to glutamate and glycine, the former could act as a potential disruptor of neurotransmitter function, interfering with this key signaling process (Myers *et al.*, 2016). *CHRNA2* and *CHRNA9*, found transversally up-regulated in all treatments at 21 days, are subunits of certain nicotinic acetylcholine receptors (nAchR) that respond to the neurotransmitter acetylcholine (Ach). The different transcriptional regulation of nAchR could be linked to the significant decrease of acetylcholinesterase (AChE) activity detected in all treatments at 7 and 21 days (Matozzo *et al.,* 2019b). While the role of glutamate and glycine in neurotransmission is yet to be elucidated in bivalve species, recent studies indicated that the neurotransmitters glutamate, Ach, and GABA may also be involved in the neuroendocrine-immune regulation network in oysters and other invertebrate species (Li *et al.*, 2016; Liu *et al.*, 2018; Wang *et al.*, 2019). Despite a stronger body of evidence on possible glyphosate neurotoxicity is

crucial, the results obtained in our study suggest possible similar neurotoxic effects for glyphosate and AMPA in bivalve species.

Of the differentially regulated transcripts seen in the common response to the chemicals investigated, particular attention should be payed to the *Heavy metal-binding protein HIP,* found more than three times up-regulated in response to AMPA and GLY at both sampling times. *HIP* has a potential function in heavy metal (e.g. Zn^{2+}, Cd^{2+}, and Cu^{2+}) and Ca^{2+} binding and has been proposed to serve in detoxification as a carrier of divalent cations in the plasma (Yin *et al.*, 2005; Hattan *et al.*, 2001). Glyphosate may form strong complexes with transition metals and metal ions (Motekaitis and Martell,1985; Undabeytia *et al.*, 2002). In our previous study, mussels exposed to high glyphosate concentrations showed a disruption of several genes involved in "response to metal ion" and "metal ion transmembrane transporter activity" (Milan *et al.*, 2018). Accordingly, the up-regulation of *HIP* proteins may be related to the chelating activity of glyphosate, in that it easily binds divalent cations, possibly leading to a disruption in Ca^{2+} homeostasis, representing one of the most reported GLY effects in vertebrates (e.g. de Liz Oliveira Cavalli *et al.*, 2013; George and Shukla, 2013; Cattani *et al.*, 2014; Uren Webster and Santos, 2015). Here, the up-regulation of HIP in AMPA-exposed mussels at both time-points suggests that this compound may have a similar mechanism of action to the one observed in GLY-exposed animals.

Over-expression of several genes involved in xenobiotic detoxification were also found, particularly after 7 days of AMPA exposure. *MGST1*, found more than 10 times up-regulated in both AMPA and GLY-exposed mussels at 7 days, plays a pivotal role in detoxification of xenobiotic compounds including organophosphorus compounds (Fujioka *et al.*, 2007; Milan *et al.*, 2011; Milan *et al.*, 2013). The *Solute carrier family 22 member 13* (*SLC22A13*), *MRP5* and *MRP9*, were also found up-regulated in response to AMPA and MIX exposed mussels (Table 2). Both multidrug resistance-associated proteins, members of the superfamily of ATP-binding cassette (ABC) transporters, and solute carrier families are extremely sensitive to environmental changes, in particular to xenobiotics. The disruption of Solute carrier family transporters was also revealed at the transcriptional level in fish (*Salmo trutta*) and shellfish species exposed to glyphosate (Uren Webster and Santos, 2015; Milan *et al.*, 2018), while the over-expression of ABC transporter genes has been related to glyphosate resistance in plants due to their participation in the excretion of toxic compounds (Nol *et al.*, 2012; Dogramacı *et al.*, 2015).

Noteworthy, several glycogen phosphorylase enzymes (*GP*; *PYG, PYGB, PYGM*) were down-regulated in GLY and MIX-exposed mussels a 21 days. These phosphorylase enzymes catalyze the rate-limiting step in glycogenolysis by releasing glucose-1-phosphate from the terminal alpha-1,4-glycosidic bond. Decreased activity of GP has previously been observed in the snails *Biomphalaria alexandrina* and *Bulinus truncatus* following Roundup and glyphosate exposure (Bakry *et al.*, 2012; Bakry *et al.*, 2015). Disruption of the carbohydrate and energy metabolisms, discussed in our previous study (Milan *et al.*, 2018), can be traced to the need to sustain energy supply in stress situations, in order to activate detoxification mechanisms and respond to indirect effects of changes occurring in host-microbiota composition.

Overall, gene expression profiling of mussels exposed to both GLY and AMPA highlight the alteration of many key molecular pathways and biological processes, reflecting both extensive compensatory responses and acute toxicity. The consequent impairment of host physiological homeostasis may have partially favored modifications on host-microbiota, leading to the spread of possible pathogenic opportunists, such as species of the *Vibrio* genus.

3.6 Conclusion

The large-scale use of glyphosate and its occurrence in aquatic ecosystems strengthens the importance to obtain information about the possible consequences of this compound on aquatic organisms. In this study, the combination of RNA-seq and 16S rRNA high-throughput sequencing allowed us to investigate host-microbiota interactions of mussels during GLY and AMPA exposure for the first time. Our data suggests that the variable capacity of bacteria to proliferate in the presence of glyphosate and the compromised physiological status of the host following exposure to AMPA and GLY may lead to significant microbiota modifications and dysbiosis. In turn, the spread of opportunistic pathogens such as *Vibrio spp.* may cause the activation of host responses mediated by increased haemocyte proliferation and changes at transcriptional level of genes involved in immune response and apoptosis regulation. Overall, our findings raise further concerns about the potential adverse effects of environmental concentrations of glyphosate and AMPA in marine species, suggesting that, in addition to the direct toxicity on host-physiology, changes occurring in host-microbial community must also be taken into consideration.

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4. The new PFAS C6O4 and its effects on marine invertebrates: first evidence of transcriptional and microbiota changes in the Manila clam *Ruditapes philippinarum*

4.1 Summary

There is growing concern for the wide use of perfluorooctanoic acid (PFOA) because of its toxic effects on the environment and on human health. A new compound – the so called C6O4 (perfluoro ([5-methoxy-1,3-dioxolan-4-yl]oxy) acetic acid) - was recently introduced as one of the alternative to traditional PFOA, however this was done without any scientific evidence of the effects of C6O4 when dispersed into the environment. Recently, the Regional Agency for the Protection of the Environment of Veneto (Italy) detected high levels of C6O4 in groundwater and in the Po river increasing the alarm for the potential effects of this chemical into the natural environment. The present study investigates for the first time the effects of C6O4 on the Manila clam *Ruditapes philippinarum* exposed to environmental realistic concentrations of C6O4 (0.1 µg/L and 1 µg/L) for 7 and 21 days. Furthermore, in order to better understand if C6O4 is a valid and less hazardous alternative to its substitute, microbial and transcriptomic alterations were also investigated in clams exposed to 1 µg/L of PFOA. Results indicate that C6O4 may cause significant perturbations to the digestive gland microbiota, likely determining the impairment of host physiological homeostasis. Despite chemical analyses suggest a 5 times lower accumulation potential of C604 as compared to PFOA in clam soft tissues, transcriptional analyses reveal several alterations of gene expression profile. A large part of the altered pathways, including immune response, apoptosis regulation, nervous system development, lipid metabolism and cell membrane metabolism is the same in C6O4 and PFOA exposed clams. In addition, clams exposed to C6O4 showed dose-dependent responses as well as possible narcotic or neurotoxic effects and reduced activation of genes involved in xenobiotic metabolism. Overall, the present study suggests that the potential risks for marine organism following environmental contamination are not reduced by replacing PFOA with C6O4. In addition, the detection of both C6O4 and PFOA into tissues of clams inhabiting the Lagoon of Venice - where there are no point sources of either compounds - recommends a similar capacity to spread throughout the environment. These results prompt the urgent need to re-evaluate the use of C6O4 as it may represent not only an environmental hazard but also a potential risk for human health.

4.2 Introduction

Per- and poly-fluoroalkyl substances (PFAS), such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), have been found in surface and groundwater worldwide and detected globally in the tissues of fish, bird, and marine mammals (Fujii et al. 2007). They result from manufacturing processes (Buck et al., 2011) and due to their extraordinary persistence, bioaccumulation tendencies and toxicological effects, PFAS represent an intrinsic threat for organisms and human health (EPA, 2017; Zheng et al., 2019). Following the industrial phase-out of PFOA, perfluoro([5-methoxy-1,3-dioxolan-4-yl]oxy) acetic acid ($C_6HF_9O_6$), commercially known as C6O4 or F-Diox acid, has been recently introduced as one of the alternative substances. C6O4, a short-chain perfluoropolyether substance, has been registered and patented by Solvay in 2014 to replace PFOA in accordance with the international EPA-PFOA Stewardship Program that proposed the abolition of PFOA and related chemicals by 2015 (ECHA, 2019; EFSA, 2014; Guruge et al., 2006; Kjølholt et al., 2015; Lu et al., 2019). The purpose was to reduce and possibly abolish environmental contamination as well as eliminating any risk for human health from PFOA, which is recognized as a major problem worldwide (EPA 2017; Bonato et al. 2020). All the chemical forms of C6O4 (*i.e.* acid, ammonium and potassium salt) are highly soluble and completely dissociate in aqueous solutions, and, due to the strong C-F

bond, they seem to be resistant to biodegradation in water (ECHA, 2019). Even though C6O4 is constantly detected in aquatic habitats (Morganti et al., submitted; ARPAV, 2020, 2019), no environmental limits have been established yet, probably also due to a crucial lack of information about its ecotoxicological effects. Publications from the Stockholm Convention stated that new generation of fluorinated substances (NGPFASs) with a short carbon chain (i.e. C4- and C6-) can replace long-chain (C≥8) homologues because potentially less hazardous, accumulative, and toxic (Chappell et al., 2020; L. Chen et al., 2018a; Kjølholt et al., 2015). In general, compared with long-chain PFAS, the short chain perfluoropolyether substances (*e.g.* GEN-X; ADONA) seem to be able to increase the solubility and the elimination speed and also appear to have a lower affinity for critical receptors in living organisms (Liu et al., 2020). However, recent studies demonstrated that these chemicals are persistent and detected in both abiotic and biotic environments (Strynar et al., 2015; Washington et al. 2020, Pan et al. 2018). Overall, while a large amount of studies confirms that finding valid substitutes to long-chain fluorinated compounds is extremely urgent due to their deleterious effects at molecular, cellular, and physiological levels (Wei et al., 2009; Li, 2010; Liu et al., 2020), knowledge about the potential effects of NGPFAs in living organisms is still extremely scarce. To date, the possible toxicity of C6O4 has been assessed only in the dossier of the European Chemicals Agency for REACH regulation, which determined the EC50 in *Daphnia magna* and *Danio rerio* respectively exposed to C6O4 for 48 and 96 hours, via ecotoxicological tests (ECHA, 2019). Given the fact that C6O4 is increasingly employed in a wide range of industrial and consumer applications, knowledge of the potential effects of C6O4 in marine organisms assumes a crucial importance to support the management and the prospective political decisions. In this context, a recent monitoring program carried out by the Regional Environmental Protection Agency of Veneto (Italy) detected relatively high levels of C6O4, up to 3200 ng/L in ground-water and about 300 ng/L in the Po river (northeast of Italy) (ARPAV, 2020, 2019). Moreover, another study revealed the presence of C6O4 in the surface water and eggs of wild bird collected nearby the Solvay production area of Spinetta Marengo (Alessandria, Piedmont, Italy) (Morganti et al. submitted). These data have greatly increased public awareness and concern for this chemical, and generated a heated public debate on the real environmental and health risks associated with C6O4. However other anthropogenic sources, such as the release from various industrial and commercial materials (*e.g.* plastic caps, products of thermoplastic industry such as sealants resistant to heat and acids) and environmental release through waste water treatment plants and atmospheric precipitation cannot be excluded in high industrialized and populated areas. The present study reports for the first time on the effects of C6O4 on a marine invertebrate, the Manila clam *Ruditapes philippinarum*. To reach such a goal, changes in transcriptional response, bioaccumulation, and microbial communities were investigated in clams exposed for 7 and 21 days to environmentally realistic concentrations of C6O4 (0.1 µg/L and 1 µg/L). Furthermore, in order to better understand whether C6O4 is a valid and less hazardous substitute of PFOA, the same interdisciplinary approach has been applied in clams exposed to 1 µg/L of PFOA. The Manila clam is an ecologically and commercially relevant edible bivalve species that inhabits lagoons and river deltas. Its choice in this study has twofold value as it is well studied as sentinel of environmental stress (Claus et al., 2016; Milan et al., 2018) and, at the same time, it represents the most important clam species cultured in Italy and the EU for human consumption.

4.3 Material and methods

4.3.1 Experimental design

Manila clams (3.64 ± 0.32 cm shell length) were collected within the Venice lagoon in February 2020. Before experiments, bivalves were acclimated in the laboratory for one week in large aquaria with aerated natural seawater (salinity of 35 ± 1‰, temperature of 12 ± 0.5 °C), previously transported from the coast to the laboratory, and were fed daily *ad libitum* with microalgae (*Isochrysis galbana*). Only healthy animals, namely

those showing reborrowing capability and extension of syphons, were used for the experiments. Duplicate glass tanks were used for each exposure concentration as well as for control animals. A total of 45 clams were maintained in each tank (90 animals for each experimental group). Perfluoro([5-methoxy-1,3-dioxolan-4-yl]oxy) acetic acid (C6O4) (CAS no. 1190931-41-9) and PFOA (CAS no. 335-67-1) were added at different concentrations, namely low (L) and high (H): 0.1 µg/L C6O4 (named C6O4_L), 1 µg/L C6O4 (named C6O4_H) and 1 µg/L PFOA (named PFOA_H) and maintained for 21 days. As the stock solution of C6O4 was prepared in methanol, in the control group (named CTRL) methanol was added at the highest concentration (20 µL/L) used in the C6O4 treatments (1 µg C6O4/L). Seawater and contaminant concentrations were renewed every 48 h. During exposure, clams were fed every 48 h by adding $6*10^6$ cells/L of microalgae solution per each tank. Samples of each experimental group were collected after 7 and 21 days of contaminants' exposure and used for subsequent analysis. Non-significant mortality of samples was observed in any group during the exposure period.

4.3.2 Chemical analyses

C6O4 and PFOA concentrations were measured three times in water samples collected from the exposure tanks: approximately 15 min after C6O4 and PFOA addition (t = 0), after 24h and 48 h (respectively before water renewal and second application of C6O4 and PFOA). Seawater samples (50 mL per tank) were collected and stored at -20°C. C6O4 and PFOA quantification in water matrices was performed by using the ISTISAN method ISS.CBA.052.REV00, developed by Istituto Superiore di Sanità (ISS) for drinking water analysis. Seawater samples were centrifuged (1 cycle at 3000 g) prior to analysis and then diluted 1:50 due to high concentration of dissolved NaCl. Samples were collected into vials, ready for direct injection into LC-MS system (ESI-). The analyses were performed using a TQ6500 mass spectrometer (Sciex, Canada) coupled with a UPLC Nexera-X2 system (Shimadzu, Kyoto, Japan). Analyses of C6O4 and PFOA bioaccumulation was carried out on soft tissue samples (10 specimens per concentration) and haemolymph (10 specimens per concentration) from clams exposed for 21 days. Haemolymph was collected from the anterior adductor muscle by a 1-mL plastic syringe, placed in Eppendorf tubes and stored on ice. Thereafter, soft tissues were excised, stored on ice, and then frozen in liquid nitrogen (along with haemolymph samples) and stored at -80°C until processing. The extraction of C6O4 and PFOA was carried out according to the method of Mazzoni et al. (2016). Briefly, samples (about 10 g of clam soft tissue and about 3.5 g of haemolymph) were spiked with 100 µL of an internal standard methanolic solution (40 µg/L, MPFAC-MXA and M3PFPeA solutions, Wellington Laboratories, Guelph, ON, Canada) and a mixture of acidified water-acetonitrile (10:90 v/v) solution was added to the spiked sample. The samples were subjected to ultra-sonication extraction, a treatment with $MgSO_4$/NaCl, freezing and centrifugation. Afterwards, the extracts were partially evaporated and then filtered by HybridSPE®-Phospholipid Ultra cartridges (Merck KGaA, Darmstadt, Germany) to eliminate phospholipids. The final extracts were analyzed by liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS) after an online purification with turbulent flow chromatography (TFC). Quantification was done using the isotopic dilution method. Limits of Detection (LODs) and Limits of Quantification (LOQs) were estimated, according to ISO Standard 6107-2: 2006, as respectively, three-fold and tenfold the standard deviation of a procedural blank. LOD and LOQ values for PFOA were 0.02 and 0.08 µg/kg ww for clam soft tissue samples and 0.07 and 0.27 µg/kg ww for haemolymph samples. LOD and LOQ values for C6O4 were 0.04 and 0.16 µg/kg ww for clam soft tissue samples and 0.14 and 0.54 µg/kg ww for haemolymph samples.

4.3.3 RNA extraction and preparation of libraries

After 7 days and 21 days, the digestive gland of 20 specimens from each experimental group was collected and stored in RNA later at -80°C until further use. For each experimental group (CTRL, C6O4_L, C6O4_H and PFOA_H) and sampling time (Day 7 and Day 21), 5 pools each composed by 4 digestive glands were prepared for total RNA extraction (RNeasy Mini Kit Qiagen, Hilden, Germany). RNA purity, concentration, and integrity of each pool were checked using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and Tape Station (Agilent, Waldbronn, Germany). RNA extracted from each pool was used for both gene expression (RNA-Seq) and microbiota analysis (16S). Library preparation for gene expression analysis was performed using Illumina TruSeq RNA Library Prep Kit. The library pools were sequenced on Illumina Novaseq 6000 (CRIBI; University of Padova) with a paired-end 2x100 bp setup obtaining a total of 1,128,494,641 paired reads (sequences available in NCBI SRA; https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA663745). For microbiota characterization, 1 µg of RNA was reverse-transcribed to cDNA using the Superscript IV kit (Invitrogen, Life Technologies, Monza, Italy). Libraries and sequencing were performed by BMR Genomics (Padova, Italy) in a 50 µL reaction starting with diluted 0.2 ng/ µL cDNA and both reverse and forward primers (10 µM) that specifically target the V3–V4 gene region of the bacterial 16S rRNA as described by Milan et al., (2018). The final libraries were then sequenced with MiSeq Illumina 300 PE. Microbiome sequencing generated 2,462,724 reads (sequences available in NCBI Sequence Read Archive SRA https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA663745).

4.3.4 Microbiome data analysis

Raw sequencing data were analysed using QIIME2 (Quantitative insights into microbial ecology 2; Bolyen et al., 2019). Reads were trimmed, filtered, and merged with cutadapt and DADA2 respectively to exclude primer sequences and, based on the nucleotide assignment, reads with quality lower than 20 (Phred Score). Features were aligned using MAFFT software (Katoh and Standley, 2013). After the quality-filter step, chimeric amplified fragments were removed and sequences merged to a total of 1,889,481 reads. Merged reads were classified with Python library scikit-learn and taxa assignment was performed using a pre-trained SILVA-database (Yilmaz et al., 2014). Final feature table and relative taxonomy were exported and analysed using CALYPSO (version 8.84) comparing all treated groups with their relative controls. In detail, to investigate changes occurring in microbial composition between CTRL and exposed clams, one-way ANOVA was carried out within each sampling time at OTU (operational taxonomic unit) and genus levels. PCoA at species level were also performed on the whole dataset as well as within each sampling time (7 and 21 days) using CALYPSO. Functional analysis of detected microbial communities was performed through PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software (Langille et al., 2013). STAMP software was used to graphically represent the obtained results.

4.3.5 Gene expression analyses

Gene expression profiles were explored through three different approaches: i) principal component analysis (PCA) as unsupervised method; *ii)* pairwise comparisons between CTRL and exposed clams (C6O4_L; C6O4_H; PFOA_H) at each sampling point (day 7 and day 21) followed by enrichment analyses; *iii)* GSEA focused on the pathways and biological processes found differentially regulated in response to chemical exposures and/or correlated to chemical concentrations in previous studies (e.g. Milan et al. 2013a; Milan et al. 2015; Iori et al. 2020). In detail, FastQC/v0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to quality check raw reads, which were subsequently trimmed to remove adaptors and low-quality reads using Trimmomatic/v0.36 (Bolger, Lohse & Usadel 2014) with default parameters. High-quality reads

were subsequently mapped against the reference transcriptome from the digestive gland, by using Kallisto/v0.46.1 (Bray et al. 2016) with default settings. The count table was generated using the "abundance_estimates_to_matrix.pl" script from the Trinity suite (Haas et al. 2013). Raw read counts were then imported into R/v3.6.0 (R Core Team 2014) and filtered: we removed contigs with less than 5 reads in at least 24 libraries (out of 40) which would contribute to background noise (Peruzza et al. 2020, Pradhan et al. 2020). Filtered reads were then normalized using the RUVs function (with parameter "k=9") from the RUVSeq/v1.18 library (Gerstner et al. 2016; Verma et al. 2020) and then normalized counts were used to perform pairwise comparisons with edgeR/v3.26.0 (Robinson, McCarthy & Smyth 2010) comparing each of the treated groups (i.e. C6O4_L, C6O4_H and PFOA_H) against the control group (i.e. CTRL) at the same time point (i.e. day_7 or day_21). 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). Of 53,476 unique sequences, 32,711 (61%) showed at least one significant match. Details and the annotation of each contig is reported in Iannello et al. (submitted). Zebrafish Ensembl IDs matching differentially expressed contigs were then used to build Venn diagrams at each sampling point. Using the SwissProt IDs to match our contig IDs, enrichment analysis was performed on differentially expressed genes using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Dennis et al., 2003; Huang et al., 2008) and considering Gene Ontology Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) databases, and KEGG pathways (KP) database. To investigate enriched GO and KP between our groups, DAVID analysis was performed considering each entire list of differentially expressed genes, as well as considering up- and down-regulated genes separately, with the following settings, gene count 2 and ease 0.05. For each pairwise comparison we further performed Gene Set Enrichment Analysis (GSEA) (Subramanian et al. 2005) to reveal, coordinated changes in gene expression within all genes. GSEA was performed using the R package clusterProfiler/v3.12.0 (Yu et al. 2012a) using custom gene sets downloaded from https://www.gsea-msigdb.org/gsea/index.jsp. Gene sets with FDR < 0.1 were deemed statistically significant.

4.4 Results

4.4.1 Chemical analyses

The concentrations of C6O4 and PFOA measured in the experimental tanks and in exposed clams are summarized in Table 1.

Table 1. PFOA e C6O4 concentrations in exposure water and in the soft tissue and haemolymph of Manila clams after 21 days of exposition.

Sample Clam soft tissue	Water nominal concentration (µg/L)	Water measured concentration (µg/L)	Sample (g ww)	Sample (mL)	PFOA (µg/kg ww)	C6O4 (µg/kg ww)	Bioaccumulation Factor (L/kg)
CTRL	0	<LOD	15.51		1.5	0.22	
C6O4_L	0.1	0.11±0.02	24.41		0.73	2.3	20
C6O4_H	1	1.01±0.07	19.41		0.58	21.1	21

Sample Clam soft tissue	Water nominal concentration (µg/L)	Water measured concentration (µg/L)	Sample (g ww)	Sample (mL)	PFOA (µg/kg ww)	C6O4 (µg/kg ww)	Bioaccumulation Factor (L/kg)
PFOA_H	1	0.93±0.31	24.84		110	0.18	119
Clam haemolymph							
CTRL	0	<LOD	3.51	3	0.30	<0.14	
C6O4_L	0.1	0.11±0.02	3.74	3.6	<0.07	<0.14	
C6O4_H	1	1.01±0.07	4.18	3.6	<0.07	9.5	
PFOA_H	1	0.93±0.31	2.91	2.5	5.2	<0.14	

During the exposure period, no loss of spiked chemicals was observed. To calculate bioaccumulation factors (BAF) in clams, the mean concentration of each chemical measured in the water was used. Concentrations in soft tissue of control specimens were 1.5 and 0.22 µg/kg ww for PFOA and C6O4, respectively and may give an indication of the background concentrations in soft tissues in the collection area. Clams accumulated both C6O4 and PFOA, but at different proportion depending on the tissue: in soft tissues PFOA is accumulated more than five times than C6O4 (BAF=119 and 21 in PFOA_H and C6O4_H, respectively), while in the haemolymph the accumulation of PFOA is only 0.6 times the accumulation of C6O4 (5.4 and 9.5 µg/kg ww for PFOA and C6O4 respectively, Table 1).

4.4.2 Microbiota characterization

Clam-associated bacterial communities consisted primarily of *Alphaproteobacteria* (51-70%), *Gammaproteobacteria* (16-39%) and *Chlamydiae* (1-11%). The PCoA analysis showed a clear separation along the x-axis (PC1= 38% of variability) between clams collected at 7 and 21 days (Fig. 1A). Within each sampling time, a weak separation at 7 days along the x-axis between treatments has been observed (22% of variability; Fig. 1C), while clams exposed to C6O4_L were clearly separated from all other treatments at 21 days (Fig. 1B and 1D). A trend of higher microbial species richness and evenness was detected across all C6O4 treatments at 21 days compared to 7 days, while no significant differences were found between control and exposed groups at both sampling times (Fig. 1E and 1F).

The full lists of significant OTUs and genera obtained for each comparison between CTRL and exposed clams are summarized in Table 2. Unexpectedly, results showed the highest number of significantly differentially represented OTUs and genera in response to the lower C6O4 concentration (C6O4_L), confirming the results obtained by PCoA (Table 2).

Table 2. Number of significant taxa obtained through one-way ANOVA analyses at OTUs and genus level comparing control group to groups exposed to C6O4 and PFOA at each sampling time (FDR<0.05). The significant taxa showing similar trend among treatments are also listed (p-value<0.05; in bolt significant taxa with FDR <0.05). The full lists of significant taxa at OTUs and genus level with corresponding p-value and FDR are reported in Bernaridni et al. 2021.

	OTU level (FDR<0.05)					
	Day 7			Day 21		
	N° Sign. OTUs	N° ↑ OTUs	N° ↓ OTUs	N° Sign. OTUs	N° ↑ OTUs	N° ↓ OTUs
C6O4_L	13	6	7	68	48	20
C6O4_H	7	5	2	2	1	1
PFOA_H	7	4	3	4	0	4
	Genus level (FDR<0.05)					
	Day 7			Day 21		
	N° Sign. Genus	N° ↑ Genus	N° ↓ Genus	N° Sign. Genus	N° ↑ Genus	N° ↓ Genus
C6O4_L	4	2	2	23	17	6
C6O4_H	4	2	2	0	0	0
PFOA_H	4	3	1	0	0	0

Significant taxa (p-value<0.05)			
	C6O4_L	C6O4_H	PFOA_H
Day 7	Vibrio Arcobacter Polaribacter_1 Methylophaga	Vibrio Arcobacter Polaribacter Methylophaga	Vibrio Polaribacter Methylotenera Methylophaga
Day 21	Polaribacter_1 Polaribacter_3 Methylotenera Profundimonas Algicola	Vibrio_otu Profundimonas Algicola	Profundimonas Algicola

In detail, the comparison between CTRL and C6O4_L revealed a total of 13 and 68 significant OTUs after 7 and 21 days, respectively. Noteworthy, at 7 days clams exposed to C6O4_L showed a significant over-representation of genera *Vibrio* and *Arcobacter*, while an opposite trend has been observed for *Methylophaga,* which found down-represented in exposed clams (FDR < 0.05). At 21 days, C6O4_L showed several significantly over-represented OTUs matching the genera *Methylotenera* and *Polaribacter*. Clams exposed to the highest C6O4 concentration (C6O4_H) showed weaker changes in comparison to C6O4_LHowever, the differentially represented taxa were similar to those detected at the lowest C6O4 concentration (i.e. *Polaribacter*, *Vibrio spp.* and *Methylophaga*). In addition, the genera *Arcobacter* and *Vibrio* were almost significantly over-represented at 7 days in C6O4_H exposed clams (p-value<0.05; FDR=0.1), confirming similar microbial dynamics to those detected in C6O4_L exposed clams. Clams exposed to PFOA_H showed similar microbial profiles to those detected in C6O4_H clams, with genera *Polaribacter* and *Methylophaga* respectively over- and under-represented at 7 days (FDR<0.05). At the same sampling time, the over representation of the genera *Vibrio* (p-value<0.05) and *Methylotenera* should also be highlighted. To conclude, at 21 days all treatments led to the down-representation of the genera *Profundimonas* and *Algicola* (p-value<0.05; Table 2).

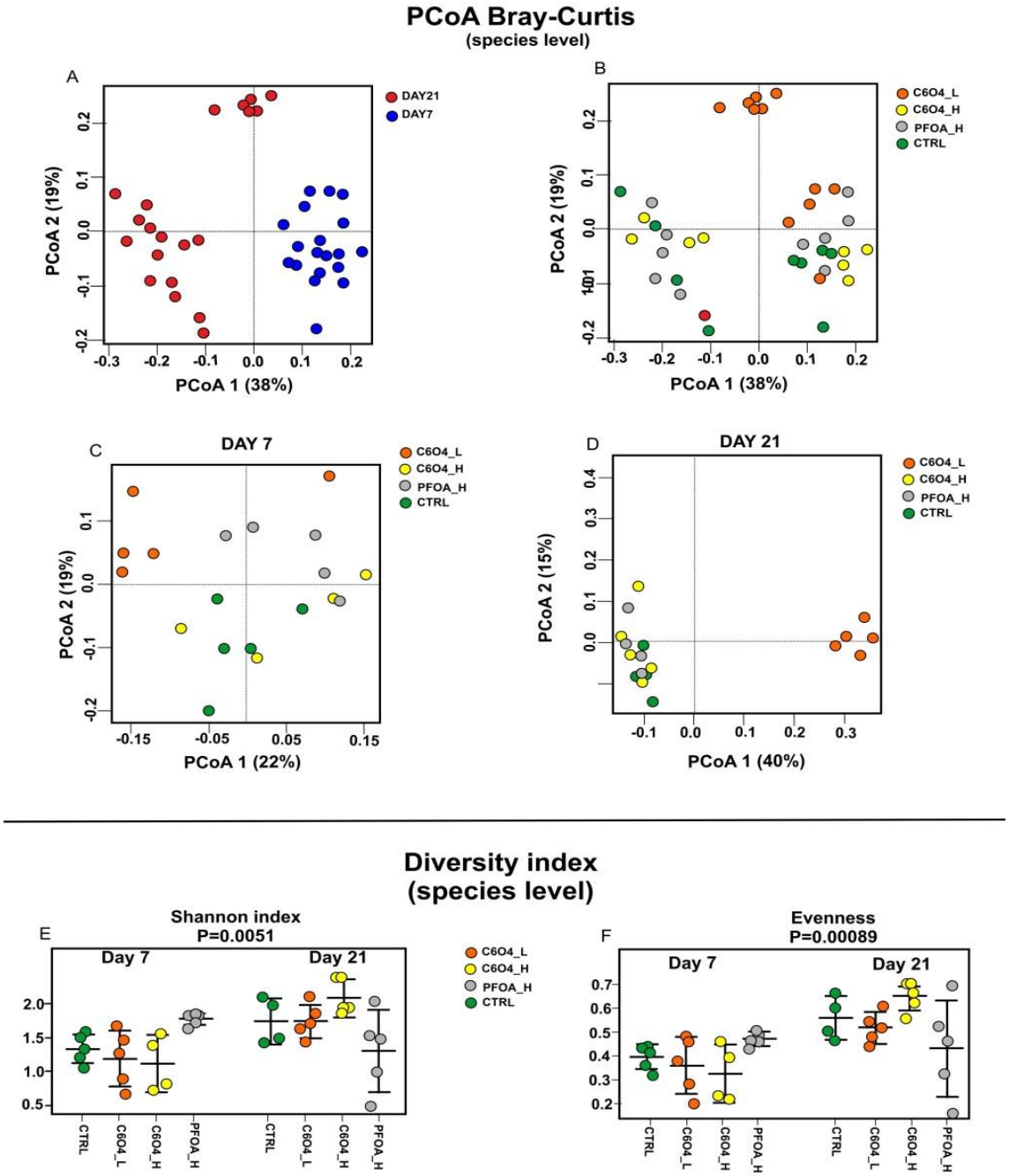

Figure 1. Principal Coordinates Analysis (PCoA) of clam digestive gland microbiota. A) Different colors indicate the two sampling time (Day 7 and Day 21); B, C, D) Different colors indicate different treatment considering the whole experiment duration (B), and each sampling time separately (C and D) ; E and F) Richness diversity index (Shannon index) and evenness for each group at 7 and 21 days.

To investigate more thoroughly the functional diversity of bacterial communities between control and exposed clams, the software PICRUST was used. Overall, analyses of the KP in exposed groups revealed changes in several microbial metabolic functions, among which “fatty acid biosynthesis” (C6O4_L at day 21 and PFOA_H at day 7), “fatty acid metabolism” (PFOA_H at day 7), “synthesis and degradation of ketone bodies” (C6O4_L at day 21 day), and “benzoate degradation” (PFOA_H at both sampling time). Noteworthy,

a significant decrease in “butanoate metabolism” was found in clams exposed to both C6O4 concentrations. The disruption of several pathways involved in the metabolism of amino acids was found in all treatments, such as “Valine, Leucine and isoleucine degradation” (in C6O4_L), “Lysine degradation” (in C6O4_L and PFOA), “Lysine biosynthesis” (in PFOA_H), “Tryptophane metabolism” (all treatments), and “Tyrosine metabolism” (in PFOA).

4.4.3 Gene expression analyses

PCA revealed a good discrimination between treatments at each sampling point (Figure 2).

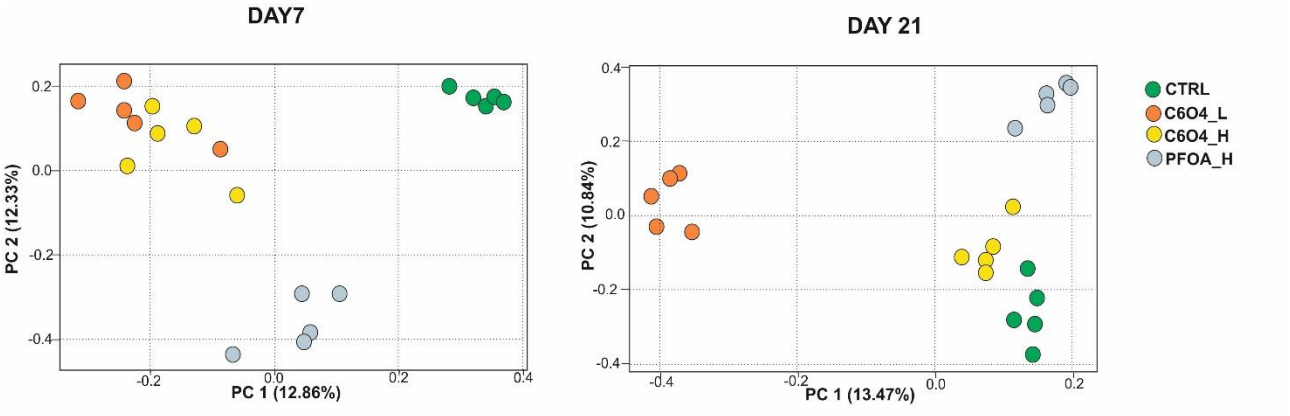

Figure 2. PCA applied to gene expression profiles at each sampling time.

After 7 days clams exposed to C6O4_H and C6O4_L clustered together, while a separation along the x- and y-axis was evident for PFOA_H exposed clams. Noteworthy, after 21 days PCA revealed a similar trend to those obtained by PCoA applied to clam’s microbiota (Fig. 1D), with a clear separation along the x-axis of clams exposed to the lower C6O4_L concentration, while along the y-axis PFOA_H and C6O4_H exposed clams appeared separated from the CTRL group. Pairwise comparisons between control and exposed clams at 7 days showed the highest number of differentially expressed genes (DEGs) in response to PFOA_H (685 DEGs) (Table 3). At 21 days the highest number of DEGs was found in clams exposed to the lowest C6O4 concentration (C6O4_L; *i.e.* 501 DEGs), while transcriptional changes in response to C6O4_H and PFOA_H decreased (475 and 339 DEGs, respectively). It should also be highlighted that, at 21 days, all treatments showed a majority of down-regulated genes compared to CTRL, with an opposite trend to that observed at day 7 (Table 3). Venn diagrams were then constructed considering *Danio rerio* gene IDs (see methods) to define the gene list of DEGs for each treatment/sampling point (Figure 3). Venn diagrams showed a total of 77 and 41 common DEGs shared between all three treatments at 7 and 21 days, respectively. Clams exposed to the two C6O4 concentrations shared 61% and 36% of DEGs at 7 and 21 days, respectively. It is worth to note that 61.7% (at 7 days) and 48.3% (at 21 days) of DEGs detected in response to C6O4_H were in common with those identified in response to PFOA_H at the correspondent sampling times. Similarly, clams exposed to the lowest C6O4 concentration shared with PFOA_H exposure 57.1% and 39.1% of DEGs at 7 and 21 days, respectively. The results obtained by the enrichment analyses are summarized in Table 3. Among the shared responses across treatments, several genes involved in “nervous system development” were commonly found differentially expressed in all treatments/sampling times (Figure 3). These results were confirmed in DAVID by the enrichment of the term “central nervous system development” (Table 3). In addition, the enrichment of the “Notch signalling pathway” was detected in clams exposed to PFOA_H and C6O4_L, including several putative notch receptors (*notc1*, *notc2*, *notc3*; Table 3). Similarly, several genes encoding members of the slit family (*slit1*, *slit2*, *slit3*), a family of secreted extracellular matrix proteins that play an

important signalling role in neural development, were up- and down-regulated in response to both C6O4 and PFOA exposure after 7 and 21 days, respectively. At both sampling times, several enriched biological processes (BP) and pathways related to immune response were found modified in all treatments.

Table 3. The upper part of the table reports the number of differentially expressed genes obtained comparing the control group to groups exposed to C6O4 and PFOA at each sampling time. Some of the enriched terms detected in response to each condition/sampling point are also reported indicating the DEGs involved in each biological processes/KEGG pathways. Genes resulted over- and under-transcribed in clams exposed to each treatment are indicated in red and green, respectively (in black genes represented by more than one significant contigs showing opposite response. The full lists of DEGs and enriched terms are reported in Bernardini et al. 2021.

	D7			D21		
	N° DEG	N° ↑	N° ↓	N° DEG	N° ↑	N° ↓
C6O4_L	579	446	133	501	247	254
C6O4_H	619	561	58	339	116	223
PFOA_H	685	500	185	475	113	362
Enriched Biological Process / KEGG pathway						
Enriched terms/functions	C6O4_L		C6O4_H		PFOA_H	
	Day 7	Day 21	Day 7	Day 21	Day 7	Day 21
Immune response/Inflammation						
Immune response/Innate immune response	TRAF3; DIAP2; VNN1; TCAM2; CO5; IIGP1; CLC4A; SRCRL; PGSC2; TIF1B	TNF15; IF44L; SAMH1; CO9; TGTP2;	CLC10; STING; DIAP2; VNN1; CO5; IIGP1; TIF1B; CLC4A; SRCRL; PGSC2	TNF15; TSP1; PXDN; IF44L; TGTP2	CLC10; TCAM2; SAMH1; IIGP1; SRCRL; CD209; DIAP2; TRAF3; SAMH1; VNN1; TIF1B; TIF1B; YM67	IF44L; TSP1; TGTP2; SAMH1
Defense response to virus	TCAM2; GBP1, IF44L; GBP3	IF44L; DMBT1; SAMH1; FCN3			SAMH1; TCAM2; GBP3, IF44L	IF44L; GBP1; SAMH1;
Cell adhesion	TENA; PTPRK; BGH3; LYAM2; PGCA; POSTN; SVEP1; CELR3; PTPRU; LYAM1; SDK1; FAT1; NPHN; SVEP1; FAT4	SVEP1; PTPRF; CELR1; MMRN1; NCAN; NPHN; MAG; TENX; PGCB;	FAT4; TENA; PTPRK; PGCA; BGH3; POSTN; SVEP1; NCAN; COCA1; LYAM1; FAT1; PGCB; PTPRT; NPHN; SVEP1;	PTPRF; EMIL1; MMRN1; PGCB; PTPRU; CELR1; SVEP1; TSP1; DSCAM; NLGN1; TENA; CSPG2; FAT4; FAT1	PTPRK; BGH3; LYAM2; SVEP1; CNTN6; PGCB; CD209; TENM; PTPRT; NPHN; SVEP1; EMIL2; CNTN4; SVEP1; ALS; CELR3; SREC; COCA1; SDK1; DSCAM	SVEP1; PTPRF; PGCB; PTPRT; MMRN1; DSCAM; TSP1; LAR; FAT4; NPHN; NOTCH; COIA1; TENR; TENX; CSPG2; TENA; LRFN3;

			CO6A5; ITA4; SVEP1; HCK;			PTPRU; FAT1; HMCN2; BGH3
Other genes involved in inflammation / immune response	C1QL3 EDAR; MRC1; BDEF; C1QL4; C1QT6;	BGBP; LGR4; C1QL3; C1QL4; CL17A; CLC4A; MRC1; STA5B; C1QT5; CLC10; DUOX1;; DUOX2; GIMAP7; IIGP5; IL6RB; LECM; LYS; MRC2; TNSF10	DMBT1; C1QL4; TNSF10; CP4FE; EDAR;	BGBP; C1QL3; BDEF; C1QL4; C1QT4; CLC4A; RO60; TRAF3; TIF1B; DTX3L; IFI27; LECH; MCLN1; C1QRF; CL17A; CLC10	MRC1; STING; IFI27; IIGP5;	C1QL3; C1QL4; IF44L; NOTC2; CL17A; BGBP; C1QL2; C1QT2; FCER2; GBP1; IIGP1; LECH; LECM; LGR4; MRC1; PAR14; TRAF3; GIMAP1; TNSF10
Apoptosis						
Apoptosis	XIAP; NTRK1; BIRC2;		BIRC2; NTRK1; XIAP; TNFSF10		TNFSF10; BIRC2; NTRK1; XIAP;	
Other genes involved in apoptosis regulation	EDAR; GA45G; DIAP2; BIR7B; TNF15; BIRC8	TNF15; GIMAP7; EDAR; GA45G; DNAS1; FGFR3; RELT	DIAP2; EDAR; CARD6	TNF15; TRAF3; IFI27; FGFR3; PAK2; RB	EDAR; DIAP2; IFI27; RELT; DIDO1; SCRIB	BIR7B; DIAP1; FGFR3; GIMAP1; TRAF3
Xenobiotic metabolism						
Xenobiotic metabolic process					ST1A4; ST1B2; CYP2B10; ST1B1; GST3	ST1A4; ST1A1 ST1B1
Sulfotransferase activity	ST1C4; ST1C2; ST1B1		ST1B1 ST1C4		ST1C4; ST1A4; ST1B2; S1C2A; ST2A1; ST1B1	ST1A4; ST1A1; ST1C4; ST2A1; S1C2A
Other genes involved in xenobiotic metabolism	MRP4	CP4P3; ST1B1; GST1;		ST1B1; CP2J5; ST1C4; CP4FN	CHST15; CP2BJ; CP2BA;	CP2BJ; CP2B2; GST1; CP4F6;

		CYP356; CP3AO			CP2B4; GSTP1	
Protein turnover						
Lysosome	SLC17A5; NAGA; GLA; CTSL		NAGA; GM2A ; GLA; CLTB; CTSL		NAGA; SLC17A5; GM2A ; GLA; NPC2; CTSL	
Protein ubiquitination			R213B; DIAP2; TIF1A; TRI33; R213A; TIF1B; TRI33		TIF1B; R213B; TRAF3; DIAP2; TIF1A; TRI33; TIF1A; R213A; TRI33	TIF1A; TRI33; R213B; TRAF3; R213A; RN213A
Cell Membrane						
Plasma membrane	MDGA1; AGRF1; AL3B1; MRC1; CELR3; SDK1; TRPA1; CRYL1; CSMD3; EDAR; EPHA5; FAT1; FAT2; FAT4; FCER2; GBP1; GP157; LIFR; LPH; LYAM1; LYAM2; MRP4; NICA; NOTC2; NPHN; PKND; PTP10; PTPRT; PTPRU; SLIT2; SPTN1; TCAM2; TNF15; TRPV1; VNN1; ZNT2; ABCA1; CD109		FAT4; MDGA1; CAD89; STING; TEN1; PTPRT; SLIT2; CD63; LPH; LPH; MKS3; SPTN1; LYAM1; FCER2; PTP10; LIFR; NCAN; PKND; CLH1; ITA4; FAT1; NETR; NETR; GP157; FAT4; LRC8D; CLC10; LRC8A; UNC5B; MCLN1; PTPRT; VNN1; GBRT; TEN2; NPHN; EDAR; NICA		MRC1; S28A1; UNC9; GSTP1; TRFR; NETR; SDK1; NETR; SREC; MDGA1; CELR3; CAD23; NOTC1; PTPRT; TENM; PLPP1; SLIT2; CD63; SPTN1; LYAM2; PTP10; HINT1; EGFR; Y4629; SAMH1; SAMH1; CD109; DOK3; LRC8D; TCAM2; CLC10; LRC8A; CNTN4; MCLN3; MCLN1; PTPRT; VNN1; TNR19; TNR19; TEN2; NPHN; EDAR; CNTN6; CAD89; FAT2	NOTC2; LAR; FAT4; NETR; FCER2; NPHN; EPHA5; FGFR3; LGR5; NOTC2; ANK1; SAMH1; SAMH1; GBP1; AGRB2; NICA; PTP10; NETR; LRFN3; PTPRU; GLUCL; NOTCH; FAT1; VNN3; SO2B1; PTPRO; MGA; NOTC1; UNC9; FLNC; LGR6; MRC1; PKND; NETR; GP157;

						DRPR; PTPRT
Integral component of plasma membrane	MRC1; ABCA1; EPHA5; FAT; FAT1; FAT2; LGR4; LPH; NOTC2; PKND; PTPRK; PTPRU; TRPV1	LGR4; NPHN; MRP3; FGFR3; LRC32; CLCA4; NOTC2; MRC2; CELR1; CEL; MRC1; NRT; SOAT; TUTL		FGFR3; FAT1; NLGN1; LGR6; S17A5; SC5A6; LGR5; LGR4; DSCAM; CELR1; PTPRU		NOTC2; LGR4; FGFR3; EPHA5; FAT1; PTPRO; MRC1; PTPRU; LGR6; SO2B1; NOTC2; LGR5; DSCAM; PKND
Glycosphingolipid biosynthesis	NAGA; GLA;		NAGA; GLA;		NAGA; GLA	
Nervous system development						
Central nervous system development		PGCB; RUNX1 NCAN; CELR1	PGCA; NCAN; PGCB; VNN1	CSPG2; PGCB; PTP10, CELR1	PTP10; PGCB; VNN1; CNTN6	CSPG2; VNN3; PTP10; ARSB; PGCB
Notch signaling pathway	FAT4; OFUT1; NICA; FAT4; NOTC2	NOTC3;; NOTC1; DLD;				NOTC3; NOTC1; NOTCH; NICA; NOTC2; FAT4

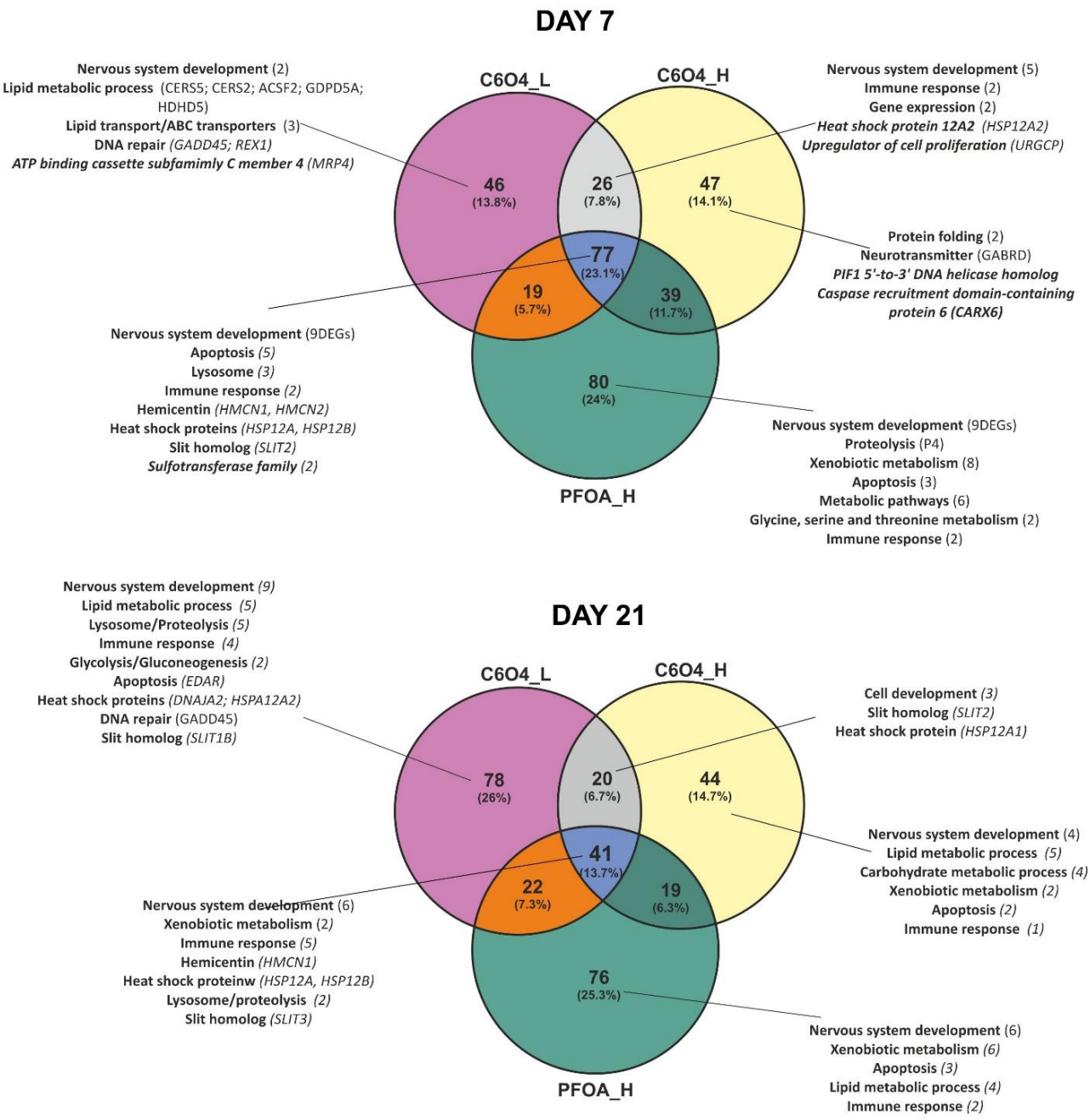

Figure 3. Venn diagrams reporting the number of differentially expressed genes for each pairwise comparison among control and exposed groups. Full lists of differentially expressed (and corresponding Danio rerio gene ID) are reported in Bernardini et al .2021 (https://doi.org/10.1016/j.envint.2021.106484).

In details, up-regulation of several genes involved in immune response, such as coding for C-type lectin (*clc4a*; *clc10*; *mrc1*), complement C1q-like proteins (*c1qt3*, *c1ql3*; *co5*, *c1*), interferon-inducible GTPase 1 (*iigp1*), soluble scavenger receptor cysteine-rich domain-containing protein SSC5D (*srcrl*), was observed after 7 days (Table 3). Conversely, after 21 days an opposite trend was observed in all treatments, with the majority of genes involved in immune responses found down-regulated (Table 3). "Cell adhesion" was the enriched BP term with the highest number of DEGs in all treatments and sampling times, including several common DEGs

across treatments. Among the shared enriched pathways, “apoptosis” deserves particular attention as this was found enriched in all treatments at 7 days. In particular, putative baculoviral IAP repeat containing 2 (*birc2*), X-linked inhibitor of apoptosis (*xiap*), death-associated inhibitor of apoptosis 2 (*diap2*) and tumor necrosis factor receptor superfamily member EDAR (*edar*) genes were up-regulated in all treatments (Table 3). In addition, at 7 days, clams exposed to the low C6O4 concentration showed also the up-regulation of growth arrest and DNA damage-inducible protein GADD45 gamma (*gadd45g*) and baculoviral IAP repeat-containing protein 7-B (*bir7b*) genes, while C6O4_H showed the up-regulation of putative caspase recruitment domain family member 6 (*card6*). Several genes involved in regulation of apoptosis, including *gadd45*, tumor necrosis factor ligand superfamily member 15 (*tnf15*) and *edar* were down-regulated at 21 days in C6O4_L, showing an opposite regulation in comparison with the 7-days sampling time (Table 3). In relation to protein turnover, the KP “Lysosome” was enriched in all treatments at 7 days, while the BP term “Protein ubiquitination” was enriched just in response to C6O4_H and PFOA_H at 7 days, in consequence of several up-regulated genes coding for E3 ubiquitin-protein ligases. The enrichment of the BP “xenobiotic metabolic process” at both PFOA_H sampling times and the up-regulation of several genes coding for Cytochrome P450 (*cyp2b19*, *cyp2j5*, *cyp2b10*, *cyp2b20*, *cyp2b4*, *cypiva2*) suggest a much stronger response toward xenobiotic metabolism in PFOA exposed clams as compared to C6O4 exposed clams. However, putative sulfotransferases (*st1c4*, *st1c2*, *st1b1*) involved in sulfotransferase activity were up-regulated also in response to both C6O4 treatments at 7 days (Table 3). Among pathways putatively involved in energy metabolism, the enriched BP term “positive regulation of adiponectin secretion” was found in all treatments and sampling times, while the BP term “negative regulation of gluconeogenesis” including putative Adiponectin (*adipo*) was enriched at both sampling times in C6O4_L and in response to PFOA_H at 21 days. Venn diagram reported in Figure 3, showed several DEGs involved in “Lipid metabolic process” in clams exposed to the lowest C6O4 concentration at 7 days, including two up-regulated genes coding for ceramide synthase (*cers5; cers2*). Up-regulated were also glycerophosphodiester phosphodiesterase domain containing 5a (*gdpd5a*) and haloacid dehalogenase like hydrolase domain containing 5 (*hdhd5*) genes, playing a role in sphingolipid metabolism and glycerophospholipid biosynthesis, respectively. Other genes involved in lipid metabolism were differentially expressed in all treatments after 21 days of exposure, among which *cers5* (up-regulated in both C6O4_H and PFOA_H treatments). Lastly, among the common response highlighted by Venn diagrams (Figure 3), several genes coding for heat shock proteins (*hsp12a*; *hsp12b*) were commonly up-regulated in all treatments at both sampling times (Figure 3). The third approach applied to gene expression analyses was based on GSEA related to each treatment/sampling time (adj. p-value<0.1). The significant pathways are reported in Table 4. First, this analysis confirmed the opposite regulation of several BP and molecular pathways at 7 *vs.* 21 days, suggesting a reduced transcriptional regulation at the end of the experiment. Second, clams exposed to C6O4_L showed a higher number of significant BP/KP compared to C6O4_H and PFOA_H. Among them, up-regulation at 7 days of “glutaminergic synapse”, “synapse”, “regulation of cell death” and “ABC transporters” should be highlighted. At 21 days an opposite regulation of “synapse” and “regulation of cell death” were found, as well as a significant down-regulation of “response to endoplasmic reticulum stress” and “cell cycle”. Third, all treatment showed a significant up-regulation of genes involved in immune response in all treatments at 7 days, confirming the results obtained with the enrichment analysis. However, the up-regulation of “innate immune response” was conserved at 21 days only in clams exposed to the lowest C6O4 concentration, while no changes or opposite regulation were found in response to C6O4_H and PFOA_H.

Table 4. List of biological processes and KEGG pathways significantly found up-regulated (red) or down-regulated (green) in each treatment/sampling time by GSEA (Adj. p-value<0.1

	DAY 7			DAY 21		
	C6O4_L	C6O4_H	PFOA_H	C6O4_L	C6O4_H	PFOA_H
XENOBIOTIC METABOLISM						
KEGG_ABC_TRANSPORTERS						
DEVELOPMENT						
ORGAN DEVELOPMENT						
STRESS RESPONSE						
GO_ION_TRANSMEMBRANE_TRANSPORT						
GO_RESPONSE_TO_ENDOPLASMIC_RETICULUM_STRESS						
NEUROTRANSMISSION/SYNAPSE						
GO_EXCITATORY_SYNAPSE						
GO_GLUTAMATERGIC_SYNAPSE						
GO_SYNAPSE						
CELL CYCLE/APOPTOSIS/CELL DEATH						
GO_CELL_CYCLE						
GO_REGULATION OF CELL CYLE						
GO_AUTOPHAGIC_CELL_DEATH						
GO_REGULATION_OF_CELL_DEATH						
SIGNALING						
GO_CANONICAL_WNT_SIGNALING_PATHWAY						
KEGG_NOTCH_SIGNALING_PATHWAY						
IMMUNE RESPONSE/INFLAMMATION						
ACTIVATION_OF_IMMUNE_RESPONSE						
DEFENSE_RESPONSE_TO_VIRUS						
GO_DEFENSE_RESPONSE_TO_VIRUS						
GO_INNATE_IMMUNE_RESPONSE						
IMMUNE_RESPONSE						
KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY						
RESPONSE_TO_BACTERIUM						
WOUND_HEALING						

4.5 Discussion

Despite still little information on ecotoxicological risk of NGPFASs is available, the prompting progressive replacement of long-chain PFAS by short-chain PFAS including C6O4 is based on the assumption that short-chain PFAS are less hazardous (Kjølholt et al., 2015). However, recent studies on short-chain PFAS revealed low-biodegradation in water, bioaccumulation in fish, invertebrates, plants and algae as well as similar acute

toxicity to their traditional counterpart long-chain PFAS (Shi et al., 2015; Liu et al., 2014; Chen et al. 2018a; Gebbink et al., 2017; Gomis et al., 2015; Gredelj et al. 2019; Munoz et al., 2019; Wang et al., 2020). Our study, thanks to an interdisciplinary approach, provides the first overview about the possible effects of C6O4 on marine species as compared to the counterpart long-chain PFOA.

4.5.1 C6O4 might reach high levels in Manila clam haemolimph and soft tissue

The average PFOA concentration detected in control clams can be considered an indicator of the PFOA concentration in the clam of the Lagoon of Venice. Even if the clams have been subject to a partial depuration in clean waters, they still show measurable concentrations of the PFAS uptaken in their environment of origin, which should represent a lower limit of contamination for clams of the Lagoon of Venice. In fact, the PFOA concentration (0.9±0.5 µg/kg ww) is lower than those measured in the Goro Lagoon (Italy) (1.6-5.8 µg/kg ww) (Mazzoni et al. 2016), which receives river water contaminated by fluoropolymer plants discharges (Valsecchi, 2015; Rusconi et al., 2015). The PFOA level observed in the Manila clams of this work is similar to PFOA concentration reported in clams from the Aichi and Kumamoto area, a populated and industrial inland sea region of Japan (0.3-1.5 µg/kg ww) (Fujii et al., 2020), whereas it is higher than that reported in clams from Nunavut, a sparsely populated area of Canada with no industrial activity (<0.4 µg/kg ww) (Ostertag et al., 2009). These findings suggest that the PFAS level in the Manila clams collected in the lagoon of Venice could be consider an indicator of the background concentration of areas impacted by anthropic activities. Similarly, average C6O4 concentration ww (0.19±0.01 µg/kg) can be considered an indicative level of the C6O4 bioaccumulation in the clams of the Lagoon of Venice suggesting that C6O4 is already diffused in the aquatic environment. The PFOA bioaccumulation factor (BAF) is in line with literature data for aquatic organisms (Valsecchi et al. 2017; RIVM, 2017). Our data suggest a 5 times lower accumulation potential of C604 as compared to PFOA in clam soft tissues. However, considering that some PFAS, including PFOA, have an immunotoxic potential in the marine bivalve *Perna viridis* - probably due to direct and indirect interactions with the haemocyte membrane (Liu et al. 2018) - we also measured PFOA and C6O4 concentrations in the haemolymph. Contrarily to what observed in soft tissues, concentration in haemolymph (at 1 µg/L of contaminants) showed values higher for C6O4 than for PFOA (Table 1), suggesting that C6O4 might reach high levels in specific tissues, potentially triggering similar toxicity to PFOA despite the lower bioaccumulation in clam whole body.

4.5.2 C6O4 and PFOA may affect the microbial communities in marine species

The digestive gland is the organ with the most important role in nutrient absorption in bivalve species. In this organ, as in vertebrates, relevant host-microbe interactions occur at mucosal interface of epithelia lining the digestive tract. Many symbiotic bacteria in the digestive gland cooperate with the immune system against pathogen and favouring energy and nutrient uptake (Allam and Pales Espinosa, 2016; Green and Barnes, 2010; Meisterhans et al., 2016). The exposure to environmental stressors, including pollutants, may lead to alterations in the gut microbiota, termed dysbiosis, often associated with a variety of pathological conditions including metabolic disorders, inflammation, and tissue degeneration (Carding et al., 2015; Green and Barnes, 2010; Snedeker and Hay, 2012; Yu, 2012b; Jin et al., 2017; Takiishi et al., 2017; Milan et al., 2019, 2018; Iori et al., 2020;). To date, few studies described the potential of NGPFASs to alter the microbiome of organisms inducing several disorders related to the microbiota activity (Chen et al., 2018b; Liu et al., 2020; Zhou et al. 2020). The present study suggests that significant changes occur in microbial communities of Manila clam's digestive gland following PFOA and C6O4 exposures. Among the most interesting results, the possible spread of *Vibrio spp.* has been observed in response to both C6O4 concentrations. *Vibrio*, a well-known genus of Gram-negative marine bacteria including species pathogenic also for humans, has been

widely described among the dominant fraction of bivalve microbiota including Manila clam (Milan et al., 2019, 2018; Vezzulli et al., 2018; Zampieri et al., 2020). Its over-representation following C6O4 exposure is in line with the opportunistic nature of *Vibrio spp.*, which are able to spread following stressful environmental conditions (*e.g*. Frydenborg et al., 2014; Green and Barnes, 2010; Li et al., 2019; Vezzulli et al., 2010), and eventually cause mass mortality events in bivalves (Le Roux et al., 2016; Alfaro et al., 2019; King et al., 2019; Milan et al., 2019). Clams exposed to the lowest C6O4 concentration, at 7 days showed also the significant over-representation of the genus *Arcobacter genus*, an emerging opportunistic pathogen frequently reported in unhealthy or moribund marine organisms such as oysters, abalones, and sponges (Tanaka et al., 2004; Fan et al. 2013; Olson et al., 2014; Lokmer and Wegner, 2015). Slight increases in *Vibrio* representation have been also observed in clams exposed to PFOA at 7 days. Despite both *Vibrio* and *Arcobacter* spp. are common members of the core microbiota of several bivalve species, their spread has been already observed following the exposure to chemicals or in bivalves inhabiting polluted areas (Iori et al., 2020; Milan et al., 2018). All treatments shared over-representation of the genus *Polaribacter* and under-representation of the genera *Methylophaga*, *Profundimonas,* and *Algicola*, suggesting similar microbial dynamics in response to the two investigated compounds (Table 2). Unexpectedly, at 21 days the most important changes in microbial communities were observed in response to the lowest C6O4 concentration. This result could be explained by possible reduced feeding rates in clams exposed to higher PFOA and C6O4 chemical concentrations, leading to weaker effects in digestive gland microbial composition. However, during controlled exposure no evident differences in excreted pseudofeces or feces were observed in any treatment. A second hypothesis is provided by hormesis possibly occurring at low concentration. Hormetic response, found in all organisms from microbes to animals, is an inverted U-shaped dose–response model, characterized by a strong effect at low doses, but reduce or no effect at high doses (Calabrese and Baldwin, 2001; Calabrese, 2005; Welshons et al., 2006). Despite hormesis responses have been frequently observed in toxicological studies, including in response to PFAS and short-chain PFAS (Cheng et al. 2011; Liu et al. 2020), additional studies are needed to better interpret the results obtained in this study, and in particular, to evaluate the potential changes in feeding rate at different C6O4 concentrations. One of the most interesting results from microbiota characterization came from the KP analysis, revealing significant functional changes in microbial metabolic functions in all treatments. Among them, pathways related to lipid and fatty acid metabolism were found enhanced in both C6O4_L and PFOA_H exposures. Noteworthy, these effects have been also observed in mice gut microbiota exposed to PFOS and to the ultrashort chain PFAS Trifluoromethanesulfonic acid (TFMS) (Lai et al., 2018; Zhou et al. 2020). Our results evidenced also the disruption of several KP related to “Amino acid metabolism” in both C6O4 and PFOA exposed clams. In this regard, a recent study reported that the dietary PFOS exposure perturbed gut metabolism in adult mice, inducing changes in amino acids and butanoate metabolism, all of which are metabolites widely recognized to be associated with inflammation and metabolic functions (Lai et al., 2018). Similarly, zebrafish exposed to the novel PFASs compound sodium *p*-perfluorous nonenoxybenzene sulfonate (OBS), one of the widely used alternatives to PFOS, demonstrated significant changes in the relative gut abundance of the genus *Vibrio*, as well as changes in various amino acids at metabolite level (Wang et al., 2020). Additional similarities in the effects induced at microbial level by PFAS were provided by the disruption of “N-glycan biosynthesis” and “benzoate degradation” in both C6O4 and PFOA exposed clams, two pathways significantly dysregulated also in mice exposed to TFMS (Zhou et al. 2020). Overall, the results here obtained suggest that both C6O4 and PFOA exposure in Manila clam lead to significant dysregulation of lipid/fatty acid and amino acids metabolism. These effects, widely reported in several model and non-model species following PFAS exposure, may also mediate host-molecular and biochemical changes (Yu et al., 2016; Alderete et al., 2019; Lin et al., 2019; Zhang et al., 2020; Chen et al., 2020; Zhou et al., 2020).

4.5.3 Similarities in the Manila clam transcriptional responses to C6O4 and PFOA

Transcriptional changes are considered the primary response to xenobiotic exposure that precedes the effective toxicity. To our knowledge, a unique study investigated the responses of Manila clam to PFAS exposure through a NGS approach, revealing transcriptional changes of several genes involved in immune response, amino acid and lipid metabolism following the exposure for 4 days to high PFOS concentrations (20 mg/L) (Zhang et al., 2020). Here, the results obtained show a high similarity in the transcriptional responses to both treatments (C6O4 and PFOA), sharing from 39% to the 62% of DEGs (Figure 3). Among others, pathways related to immune response, apoptosis regulation, nervous system development, lipid metabolism and cell membrane affected by both C6O4 and PFOA exposures, deserve particular attention. The sub-epithelial tissues along the digestive tract are among the most haemocyte-rich tissues in molluscs. Both PFOA and C6O4 lead to significant changes of many genes playing key roles in bivalve immune response (Table 3). In addition, considering that the defence mechanism of phagocytosis and spontaneous cytotoxicity relies on effective cell attachment, the enrichment of "Cell adhesion", representing the BP term with the highest number of DEGs in all treatments, should be highlighted. The overall up-regulation of immune-related genes evidenced at 7 days could be in part related to the spread, in the early phase of chemical exposures, of the two opportunistic pathogen genera *Vibrio* and *Arcobacter* described above. However, immune modulations following PFAS exposures have been already widely described in model and non-model species, including clams (Guruge et al., 2006; Dong et al., 2009; Grandjean and Budtz-Jørgensen, 2013; Zhang et al., 2020). Among the most interesting studies, it is worth mentioning the work by Liu and Gin (2018) on *Perna viridis* investigating the potential immunotoxicity of four commonly detected PFAS. Results from this study revealed reduced haemocyte cell viability and suppression of the immune function by up to 50% of normal performance, underlying the immunotoxic potential of PFASs. Additional evidence from the literature suggests that PFAS can cause suppression of genes related to immunity and specially to cell adhesion (Cui et al., 2009; Guruge et al., 2006), supporting the results obtained in the present work. In fact, both C6O4_H and PFOA_H exposed clams showed at 21 days an increased number of down-regulated genes involved in immunity and cell adhesion. The most interesting hypothesis explaining how PFAS cause immunotoxicity is the possible intercalation of these chemicals in biological membranes, leading to membrane perturbation in cells and cellular organelles that are involved in an organism's innate defence system (Hu et al., 2003; Liu and Gin, 2018). In our study, membrane perturbations in both C6O4 and PFOA exposed clams is suggested by the enrichment of several BP and CC terms such as "plasma membrane", "integral component of plasma membrane" and "glycosphingolipid biosynthesis", as well as by several DEGs involved in "glycerophospholipid biosynthesis" and by the up-regulation of ceramide synthase (*cers5; cers2*) playing a central role in sphingolipid metabolism (Mullen *et al.,* 2012; Šabović *et al.* 2020). It should be noted that PFAS exposure has been associated with dysregulated lipid metabolism also in humans (e.g. Chen et al., 2020). Possible effects of PFOA and C6O4 at the level of nervous system development are suggested by the enrichment of the BP "central nervous system development", as well as by several DEGs involved in "nervous system development" (Table 3 and Figure 3). Additional evidence was provided by the enrichment of "Notch signalling pathway", promoting the proliferative signalling during neurogenesis, and by the disruption of genes encoding members of the *slit* family, involved in neuronal development and differentiation in vertebrates. However, considering that our study was focused on Manila clam's digestive gland, additional studies focused on other tissues are needed to provide a wider perspective on the possible effects of these chemicals on key molecular pathways related to the nervous system. Just few genes and enriched pathways involving energy metabolism were found modified in all treatments. Among them, particular attention should be deserved to the *adiponectin* (*adipo*) gene, that resulted up-regulated in response to C6O4_L at both sampling times and to PFOA_H at 21 days. The *adipo* gene is associated to fatty acid oxidation, regulation of glucose metabolism, inflammatory processes and anti-apoptosis in vertebrate species (Berg et al., 2002; Tsatsanis et al., 2005; Park et al., 2007;). In invertebrate species *adipo* seems to play vital roles in energy metabolism and autophagy (Zhu et al., 2008; Kwak et al., 2013; Chen et al., 2019). In addition, a recent study

focused on the oyster *Crassostrea gigas* suggested that *Adiponectin receptor* plays a key role in the immune response of oysters by regulating the expressions of inflammatory cytokines and haemocyte apoptosis (Ge et al., 2020). While the role of *adipo* in the apoptotic process is not yet clear in invertebrates, the role of programmed cell death in homeostasis maintenance and immune defence have been widely described also in mollusc species (Romero et al., 2015; Nguyen et al., 2019;). Here, a largely overlapping profile of apoptosis regulation was observed in both treatments of experimental clams (Table 3). This profile is also in agreement with the results obtained in other bivalve species exposed to pollutants (Pruski, 2002; Sokolova, 2004; Romero et al., 2011;). In particular, up-regulation of caspases, *birc* and *xiap* genes were reported in mussels and clams in response to a variety of different chemicals (*e.g.* NSAIDs, heavy metals, and glyphosate among others) (Milan et al., 2016, 2013b; Mezzelani et al., 2018; Iori et al., 2020). The transcriptional changes of several genes involved in apoptosis is likely to be a generic response to chemical/environmental stress rather than a specific mechanism activated in response to PFAS or short-chain PFAS exposure. Similarly, the up-regulation of several genes coding for heat shock proteins (*hsp12a*, *hsp12b*) may represent a generic reaction to chemical stressors (Monari et al., 2011). However, their up-regulation also at lower concentration of C6O4, as well as the dysfunction of the other key molecular pathways here described, represents a warning signal pointing to the potential toxicity of this new short-chain PFAS.

4.5.4 Manila clam shows unexpected dose-dependent responses to C6O4

While clams exposed to the two concentrations of C6O4 showed similar transcriptional responses in the early phase of chemical exposures, at 21 days a different pattern appeared. The clear separation of clams exposed to the lowest concentration of C6O4, observed also in PCA, can be related to the general slowdown of gene transcription observed at 21 days just in clams exposed to the highest C6O4 and PFOA concentrations (65% and 76% of down-regulated genes, respectively).

Overall, these different transcriptional trends can be explained *i)* by hormesis occurring at low concentration or *ii)* as a response to highest chemical concentrations triggering cells to start a coordinated response to protect themselves from damage through a rapid down-regulation of many genes in a process called Stress-Induced Transcriptional Attenuation (SITA; Aprile-Garcia et al., 2019). Down-regulation of many genes in response to triggers of cellular stress, such as high temperatures, lack of nutrients or infections, is a phenomenon that is poorly understood, although described in many different species such *Drosophila melanogaster*, mouse and human cells (Gasch et al., 2000; Mahat et al., 2016; Duarte et al., 2016). Noteworthy, a recent study that investigated SITA as a consequence of heat shock as a stress model, suggested that protein ubiquitination plays a key role in this transcriptional down-regulation program. In our study, "protein ubiquitination" BP term was enriched just in response to the highest concentrations of C6O4 and PFOA, represented at 7 days by several up-regulated genes including E3 ubiquitin-protein ligase as *trim33* and its homologs *trim24* and *trim28*, thought to be transcriptional corepressors (Agricola et al., 2011).

Among the most evident differences in the transcriptional responses to the two C6O4 concentrations there was also the conserved up-regulation of several KG and BP related to immune response in clams exposed to the lower C6O4 concentration at 21 days (Table 4). This result could be explained by the possibility to keep an "active" gene transcription at lower C6O4 concentration, as well as by stronger host-microbiota interactions that may play a key role in the overall modulation of host-immune responses (Lai et al., 2018; Wang et al., 2020).

4.5.5 Specific hazards derived by C6O4 and PFOA exposures

While most of the disrupted molecular pathways are common in C6O4 and PFOA exposed clams, specific response to chemical exposure were also observed. Among them, clams exposed to PFOA showed a much higher change in genes involved in xenobiotic metabolism. This is demonstrated by the enriched term "xenobiotic metabolic process", by the up-regulation of several genes belonging to cytochrome P450 superfamily (CYP450), and by the highest number of up-regulated genes belonging to sulfotransferase family (*sult*) detected in PFOA exposed clams at both sampling times. While CYP450 plays a role in phase I drug metabolism, *sult* is a family of phase II detoxification enzymes that are involved in the homoeostasis of endogenous compounds, as well as in protection against xenobiotics (Negishi et al., 2001). Up- regulation of *sults* in bivalve species was reported in several studies of controlled exposures to chemicals (Guo et al., 2017; Lavado et al., 2006; Milan et al., 2013b) as well in populations inhabiting polluted areas (Milan et al., 2015, 2013a). On the one hand, this result suggests different capacity of clam detoxification enzymes to be induced by C6O4 and PFOA. On the other hand, the marked activation of the detoxification systems following PFOA exposure could be able to limit the effects highlighted in other biological processes described below.

In particular, possible narcotic or neurotoxic effects specific to clams exposed to C6O4 were suggested by the gene expression analysis. First, GSEA revealed significant changes for GO terms related to synapse only in C6O4 treated clams, as "glutaminergic synapse", "synapse" and "excitatory synapse". Second, transcriptional changes of acetylcholinesterase (*ache*) and neuroligin 3 (*nlgn3*) were exclusively reported in clams exposed to the lowest C6O4 concentration. Similarly, gamma-aminobutyric acid receptor subunit theta (*gabrq*), was over expressed in the C6O4_H group at 7 days, whereas the same experimental group showed down-regulation of neuroligin 4 (*nlgn4*) at 21 days. *nlgn3* and *nlgn4* belong to a family of neuronal cell surface proteins exhibiting synaptogenic activity (Chih, 2005; Nam and Chen, 2005). Homologues of mammals NLGN have been identified in the nervous system of many invertebrates suggesting a possible role in synaptic formation (Knight et al., 2011). While GABRQ mediates the fastest inhibitory synaptic transmission in the central nervous system, AChE is an enzyme playing a key role in the termination of synaptic transmission through the breakdown of acetylcholine and of other choline esters that function as neurotransmitters. It was demonstrated that AChE is inhibited by several chemicals such as pesticides and some metals (Gill et al., 1991; Mora et al., 1999; Key and Fulton, 2002; Dailianis et al., 2003), and it was also inhibited in clams inhabiting polluted areas (Matozzo et al., 2005). The literature suggests that some PFAS may be neurotoxic. In particular, relationships between PFAS exposures and neurotransmitter alterations (Dassuncao et al., 2019), neuronal differentiation (Slotkin et al., 2008), synapses (Lee and Viberg, 2013) and potential motor deficit (Chen et al., 2014; Onishchenko et al., 2011) were already described in invertebrate species. Noteworthy, Chen and colleagues (2018b) recently demonstrated the perturbation of multiple neural signalling processes, including cholinergic, glutamatergic, and GABAergic systems as the result of medaka embryos exposure to environmentally realistic concentrations of perfluorobutane sulfonate (PFBS), an industrial alternative to PFOS (Chen et al., 2018b). Additional specific transcriptional responses to C6O4_L exposure were the up-regulation of *gadd45*, and the down-regulation at 21 days of the GO "Cell Cycle" and "Cell Cycle regulation". GADD45 belong to a group of regulatory molecules that primarily protect cells to ensure survival under stressful conditions by cell cycle arrest, DNA repair and, ultimately, apoptosis activation (Fornace et al., 1989; Hollander et al., 1999). A role of *gadd45g* in cell-cycle could be through its interaction and inhibition of the kinase activity of the Cdk1/cyclin B1 complex (Vairapandi et al., 2002). Accordingly, the transcriptional down-regulation of genes involved in cell cycle regulation of clams exposed to C6O4_L at 21 days may reflect potential cell cycle arrest mediated by GADD45G as a result of cellular stress.

4.6 Conclusion

Following the need to find valid substitutes, C6O4 was recently introduced as one of the alternatives to PFOA, despite the lack of any scientific evidence on the effects of this new chemical when dispersed into the environment. By combining chemical and molecular analyses, the present study provided the first evidence of possible environmental threats of this new compound. The detection of both C6O4 and PFOA into clam tissues of the Lagoon of Venice, despite the absence of local sources of these chemicals, suggests their potential ability to spread throughout the environment and, consequently, their possible entry into the trophic chain. Molecular analyses suggest that C6O4 may cause significant perturbations to the digestive gland microbiota and alter pathways at transcriptional level, including immune response, apoptosis regulation, nervous system development, lipid metabolism and cell membrane metabolism. In addition, clams exposed to C6O4 showed possible narcotic or neurotoxic effects and reduced activation of genes involved in xenobiotic metabolism. Despite PFOA showed higher accumulation potential than C6O4 in soft tissues, our results suggest that the potential effects in marine organisms following environmental contamination are not reduced by replacing PFOA with C6O4. It appears evident the urgent need of extending our knowledge on C6O4 bioaccumulation in natural populations of marine organisms as well as on the potential ways of uptake and their potential depuration ability. Further studies on the effects of this chemical particularly on edible marine species will shed more light not only on the possible environmental hazard but also on risk for human health.

4.7 References

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5. New compounds, old problems. The case of C6O4 - a substitute of PFOA - and its effects to the clam *Ruditapes philippinarum*

5.1 Summary

C6O4 (difluoro{[2,2,4,5-tetrafluoro-5-(trifluoromethoxy)-1,3-dioxolan-4-yl]oxy}acetic acid) is a new surfactant and emulsifier used as a substitute of perfluorooctanoic acid (PFOA). Recently, C6O4 has been detected in aquatic environments, but, at present, no information concerning the effects of C6O4 on aquatic species, such as bivalves, are available in the literature. Therefore, in this study we evaluated for the first time the effects of C6O4 (0.1 and 1 µg/L) and PFOA (1 µg/L) to the clam *Ruditapes philippinarum*. Short-term (7 days) and long-term (21 days) exposures of clams to the two compounds were carried out and numerous biomarkers were measured in haemocytes/haemolymph, as well as in gills and digestive gland. The MANOVA analysis demonstrated statistically significant effects of the independent variables "treatment", "time" and "treatment-time interaction" on the whole dataset of biomarker responses. The two-way ANOVA analysis performed for each biomarker response indicated that the two compounds affected most of the cellular and tissue parameters measured. Despite preliminary, the results obtained suggested that C6O4 - similarly to PFOA - can affect both cellular and biochemical parameters of clams.

5.2 Introduction

Per- and poly-fluoroalkyl substances (PFASs) are used since 1950s and include more than 7000 compounds characterised by fluorine-carbon bonds (Buck et al., 2011, Johnson et al., 2021). PFASs are mainly used as surfactants or repellents of both oil and water in a wide spectrum of applications, such as fluoropolymer industry, textile and food packaging industry, in aqueous film forming foam and in metal-plating industries (Lindstrom et al., 2011, Sunderland et al., 2019). Due to the highly resistant chemical bonds, such compounds are classified as persistent organic pollutants (POPs). Indeed, PFASs are highly resistant to photolysis, hydrolysis, chemical and thermal degradation (Sznajder-Katarzyńska et al., 2019). As for biodegradation of PFASs, there is evidence for biodegradation of some compounds, even if such process is incomplete and may not result in mineralisation (Parsons et al., 2008).

The most used long chain PFASs are perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) that have been respectively included in Annex A (elimination) and in Annex B (reduction of its production) of Stockholm Convention (UNEP, 2009a, UNEP, 2019b). In addition, the International Agency for Research on Cancer (IARC) classified PFOA as a possible carcinogen to humans (IARC, 2017). PFOA, which is mainly used as wetting and dispersion agent in polytetrafluoroethylene (PTFE) production (Pistocchi and Loos, 2009), has been phased out by the USA chemical industry since 2015 (Mueller and Yingling, 2017). PFOA direct emissions into the environment have been estimated in 30 tons/year (1995–2024), even if PFOA can also originate from fluorotelomer alcohols (FTOHs) degradation (Van Zelm et al., 2008, Wallington et al., 2006). PFOA has been detected worldwide in many water bodies. It has been recorded not only downstream of fluoropolymer manufactories (up to 6500 ng/L and 0.97 mg/L in Italy and China, respectively) (Rusconi et al., 2015, Wang et al., 2016) and in Waste Water Treatment Plants (WWTPs) (up to 12.7 µg/l) (Vo et al., 2020), but also in rivers of South Africa (390 ng/L), Italy (303 ng/L), China (260 ng/L) (So et al., 2007, Mudumbi et al., 2014, Castiglioni et al., 2015) and in lakes of China (10.6–36.7 ng/L) (Yang et al., 2011) and USA (10–64) ng/L (Sinclair et al., 2006). PFOA can reach the sea through the water flux, with an estimated river discharge to coastal areas in Europe of about 30 tons/year in 2007 (Pistocchi and Loos, 2009). In coastal marine ecosystems, PFOA reached levels up to 2.15 ng/L in Orbetello lagoon, 19.4 ng/L and 26 ng/L in the Lagoon of Venice and Po estuary (Polesello et al., 2011), 68.6 ng/L along Korean coasts (Naile et al., 2010), 320 ng/L in Korean seawaters (So

et al., 2004), 15.3 ng/L in China coastal areas and 192 ng/L in Tokyo bay (Yamashita et al., 2005). Furthermore, PFOA has been detected in the North Sea (up to 4 ng/L) (Ahrens et al., 2009a, Ahrens et al., 2009b), in Western Pacific Ocean (up to 500 pg/L) (Yamashita et al., 2008), in North Atlantic Ocean (229 pg/L) (Ahrens et al., 2009a; 2009b) and in Antarctic seawaters (up to 15 ng/L) (Cai et al., 2012). As regards the effects of PFOA, data on the molecular mechanisms and signalling pathways of PFOA-induced toxicity in animals and humans have been summarised in the review of Li et al. (2017).

Perfluoroalkyl ether carboxylic acids (PFECAs) have been recently introduced in replacement to PFOA as polymerisation aids in production of perfluoropolymers (Wang et al., 2020a, Wang et al., 2020b). These compounds contain oxygens in their carbon chain (ether group), but maintain similar chemical properties to those of PFOA (Wang et al., 2015, Gomis et al., 2015). Although PFECAs have been poorly researched in aquatic ecosystems and investigated from an ecotoxicological point of view, they are considered as emerging environmental contaminants (Wang et al., 2017, Gebreab et al., 2020). Strynar et al. (2015) demonstrated the presence of a series of perfluorinated ether carboxylic and sulphonic acids, along with traditional PFASs, in surface water in North Carolina (USA). The compounds consist of a homologous series of per- and polyfluorinated compounds with repeating units of CF_2 or CF_2O subunits.

A recently used PFECASs is C6O4 (difluoro{[2,2,4,5-tetrafluoro-5-(trifluoromethoxy)-1,3-dioxolan-4-yl]oxy}acetic acid) (Fig. 1). C6O4 is classified as perfluoroether non-polymer with saturated bonds available in four different forms: *i*) acid form (CAS: 1190931-41-9), *ii*) potassium salt form (CAS: 1190931-39-5), *iii*) ammonium salt form (CAS:1190931-27-1) and *iv*) reduced alcohol form (CAS: 1190931-34-0) (ECHA, 2021, Wang et al., 2020a; 2020b). C6O4 has been registered by MITENI S.p.A. (now inactive) and by Solvay (active registrant) (ECHA, 2021). ECHA registered dossier reports a total tonnage band of 10–100 ton per year (ECHA, 2021).

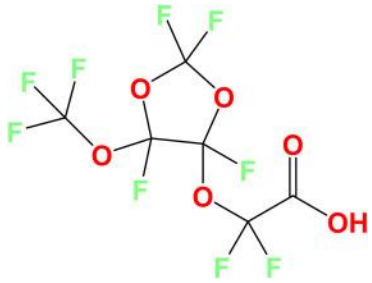

Figure 1. Structure of C6O4 ((difluoro{[2,2,4,5-tetrafluoro-5-(trifluoromethoxy)-1,3-dioxolan-4-yl]oxy}acetic acid).

As ammonium salt, C6O4 is used as a polymer production aid during the manufacture of fluoropolymers as substitute of PFOA (EFSA, 2014). C6O4 has been detected in industrial discharge, ground and surface water in Italy. A range of 50–100 µg/L was measured in the discharge of the perfluoropolymer plant of Solvay, located in the Western sector of the Po River valley (Morganti et al., 2021). C604 was also found in the river Bormida, a Po tributary, downstream from the Solvay factory (Wang et al., 2020a; 2020b), and it was detected by Environmental Agency of the Veneto Region in the Po River waters close at its mouth with ranging concentration from <LOD to 1190 ng/L (ARPAV, 2020). The same Agency found C6O4 in aquifer underneath the MITENI production site with a maximum concentration of 3265 ng/L (ARPAV, 2020).

The recent replacement of PFOA with C6O4 and the limited information on the effects of C6O4 to aquatic species (ECHA, 2021) pose several questions on C6O4 ecotoxicological profile. Consequently, in this study - that is a continuation of our previous one (Bernardini et al., 2021) - we evaluated for the first time the effects of C6O4 and PFOA on cellular and biochemical parameters of the clam *Ruditapes philippinarum,* a bivalve species widely used in ecotoxicological investigations. The hypotheses we tested were *i*) whether a short-

term (7 days) or a long-term (21 days) exposure to C6O4 could pose a potential risk to marine bivalves and *ii*) whether the effects induced by C6O4 are comparable to those caused by PFOA.

5.3 Materials and methods

5.3.1 Clam acclimation and exposure

R. philippinarum specimens (3.64 ± 0.32 cm shell length) were sampled in a licensed area for bivalve culture in the southern basin of the Lagoon of Venice (Italy) and acclimated for 7 days in large aquaria filled with a sandy bottom and aerated seawater (salinity of 35 ± 1, temperature of 12 ± 0.5 °C). As food supply, a suspension of the microalgae *Isochrysis galbana* was provided ad libitum.

Two stock solutions (50 mg/L) of C6O4 (CAS no. 1190931-41-9) (Wellington Laboratories, Canada; purity: > 98%) and PFOA (CAS no. 3825-26-1) (Sigma-Aldrich, Milano, Italy; purity: > 98%) were prepared in methanol. Clams (90 in total per each concentration tested) were exposed for 7 and 21 days to 0.1 and 1 µg/L of C6O4 in 30 L glass tanks (two tanks per each concentration, with 45 clams per tank) without a sandy bottom. To compare the potential toxicity of C6O4 with that of PFOA, clams were also exposed to PFOA at the highest concentration tested in C6O4-exposed group, namely 1 µg/L. A control was running in parallel by adding methanol in seawater at a concentration of 20 µL/L. Based on our preliminary observations (data not published), such concentration of methanol was unable to produce significant alterations in biomarker responses of clams with respect to a control condition without solvent. In addition, a previous study demonstrated that methanol could cause lethal or sub-lethal effects in aquatic species, such as fish and invertebrates, at relatively high concentrations, in the order of grams or milligrams per litres (Kaviraj et al., 2004). That is why we decided to prepare only one control (seawater + methanol). Seawater, working solutions of C6O4 and PFOA and food supply (microalgae suspension at an initial concentration of about 9×10^8 cells/L) in exposure tanks were renewed every 48 h.

Actual concentrations of C6O4 and PFOA in seawater samples from experimental tanks, as well as bioaccumulation of the two compounds in clams, were also evaluated and the results obtained are reported in Bernardini et al. (2021). A synopsis of the used chemicals, analytical methods and results is reported in supplementary material (S1).

5.3.2 Tissue collection

Haemolymph was collected from the anterior adductor muscle by a 1-mL plastic syringe and stored in Eppendorf tubes at 4 °C. At each sampling time, 5 pools of haemolymph (from four clams each) from each experimental condition were prepared. A volume of pooled haemolymph was immediately used to measure total haemocyte count (THC), haemocyte diameter and volume, haemocyte proliferation (XTT) and lactate dehydrogenase (LDH) activity. The remaining part of pooled haemolymph was then centrifuged at 780 × *g* for 10 min, the supernatant was discharged, while the pellets (=haemocytes) were resuspended in distilled water to obtain haemocyte lysate (HL) samples. To this aim, samples of haemocytes resuspended in distilled water were vortexed for 30 s and centrifuged at 780 × *g* for 10 min at room temperature. The supernatant, corresponding to HL, was then collected, frozen in liquid nitrogen and stored at − 80 °C until analyses (lysozyme activity assay).

Gills and digestive gland from clams were then excised, pooled to obtain five different pools of four clams each, divided in aliquots, frozen in liquid nitrogen, and stored at − 80 °C until analyses.

5.3.3 Haemocyte parameters

THC, as well as haemocyte diameter and volume, were determined using a Scepter™ 2.0 Automated Cell Counter (Millipore, FL, USA). Briefly, 20 µL of haemolymph were added to 2 mL of Coulter Isoton II diluent. The THC was expressed as the number of haemocytes (10^7)/mL of haemolymph, while haemocyte diameter and volume were expressed in µm and picolitres (pL), respectively.

Haemocyte proliferation was evaluated using the *Cell proliferation* Kit II, Roche (a commercial kit), as described in our previous study (Matozzo et al., 2012). In brief, a volume of 200 µL of the mixture provided by the kit was added to 400 µL of pooled haemolymph and incubated for 4 h in a dark humidified chamber. The absorbance at 450 nm was then recorded using a Beckman 730 spectrophotometer. The results were normalised to THC values of each experimental groups and expressed as optical density (OD) at 450 nm.

The commercial *Cytotoxicity Detection* Kit, (Roche) was used to measure lactate dehydrogenase activity (LDH) in cell-free haemolymph (CFH). Pooled haemolymph (500 µL) from each experimental condition was centrifuged at 780 x g for 10 min, and the supernatant (=CFH) was then collected for the assay following the manufacturer's instructions. The results were expressed as optical density (OD) at 490 nm.

The lysozyme activity was measured in haemocyte lysate (HL) from pooled haemolymph (500 µL). HL was obtained as described above. Briefly, 50 µL of HL was added to 950 µL of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma) in 66 mM phosphate buffer (pH 6.2), and the decrease in absorbance (ΔA/min) was continuously recorded at 450 nm for 3 min at room temperature. The results were expressed as µg lysozyme/mg of protein. Protein concentrations in the HL were quantified according to Bradford (1976).

5.3.4 Gill and digestive gland enzyme activity assays

Gills and digestive gland samples were homogenised at 4 °C with an Ultra-Turrax homogeniser (model T8 basic, IKA) in four volumes of 10 mM Tris-HCl buffer, pH 7.5, containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA and protease inhibitor cocktail (1:10 v/v) (Sigma-Aldrich), and centrifuged at 12,000*g* for 30 min at 4 °C. Supernatants (SN) were collected for analyses. The protein concentration in SN samples was quantified according to Bradford (1976).

Total superoxide dismutase (SOD) activity was measured in both gills and digestive gland in triplicate using the xanthine oxidase/cytochrome c method proposed by Crapo et al. (1978). Enzyme activity was expressed as U/mg protein, one unit of SOD has been defined as the amount of sample causing 50% inhibition under the assay conditions.

Catalase (CAT) activity was measured in gills and digestive gland SN in triplicate following the method proposed by Aebi (1984). The enzyme activity in a volume of 30 µL of tissue SN were measured at 240 nm and expressed as U/mg protein; one unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 µmol of H_2O_2/min.

The method of Ellman et al. (1961) was used to measure acetylcholinesterase (AChE) activity in gill SN (25 µL), following the colorimetric reaction between acetylthiocholine and the reagent dithiobisnitrobenzoate. Changes in absorbance were then recorded at 405 nm for 5 min on a microplate reader at room temperature. The results were expressed as nmol/min/mg of protein.

Glutathione S-transferase (GST) activity was measured in digestive gland SN according to the method described in Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates. GST activity was expressed as nmol/min/mg protein.

Glutathione reductase (GR) activity was evaluated according to Smith et al. (1988), by measuring the (5-thio (2-nitrobenzoic acid)) TNB production at 412 nm. The enzyme activity was expressed as U/mg protein.

Oxidative damage in gills and digestive gland was measured through both protein carbonyl content (PCC) and the DNA strand breaks assays. Briefly, PCC was measured in duplicate using the method of Mecocci et al. (1999) following the reaction with 2,4-dinitrophenylhydrazide (DNPH). Results were expressed as nmol carbonyl group/mg protein. The DNA strand breaks were measured using a fluorescence method adapted from an alkaline precipitation assay (Olive, 1988). Fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer at 360 nm (excitation) and 450 nm (emission) against blanks. Salmon sperm genomic DNA standards were used for DNA calibration, and the results were expressed as µg/mg protein.

5.3.5 Statistical analysis

The normal distribution of data (Shapiro-Wilk's test) and the homogeneity of the variances (Bartlett's test) were assessed. The MANOVA analysis was performed to highlight the effects of the factors "treatment", "time" and "treatment-time interaction" on the whole dataset of the biomarker responses. For each biomarker, results were compared using a two-way ANOVA (with treatment and time as predictors), followed by Tukey's HSD post-hoc test for pairwise comparisons. All results are expressed as means ± standard error (n = 5).

A canonical correlation analysis (CCA) was also performed. The factors "treatment", "time" and their interaction have been included in the first set, while the measured cellular and biochemical parameters are used in the second one.

The software packages Statistica 13.4 (TIBCO Software Inc.) and R (R Core Team, 2020, Austria) with the CCA package (González and Déjean, 2012) and the r41sqrt10 package (Finos, 2020) were used for the statistical analyses.

5.4 Results

No clam mortality was recorded during experiments. Interestingly, during exposure at least five clams treated with 0.1 µg/L of C6O4 showed foot cut-off, clams being unable to withdraw it before shell closure (Fig. 2).

Figure 2. Clams exposed to C6O4 0.1 µg/L. Two images showing clams with almost cut foot (black circle) that remained out of the shell after its closure.

A statistically significant effect of the independent variables "treatment" (MANOVA: $F_{(54,51)} = 3.206$, $p < 0.001$) "time" (MANOVA: $F_{(18,15)} = 48.87$, $p < 0.001$) and "treatment-time interaction" (MANOVA: $F_{(54,51)} = 3.789$, $p < 0.001$) on the whole dataset of dependent variables (biomarker responses) were recorded.

As for graphs, asterisks denoting statistically significant variations in biomarker responses between control and treated groups at each sampling time were reported only when a significant effect of the factors “treatment” and/or “treatment-time interaction” was revealed by the two-way ANOVA analysis.

5.4.1 Haemocyte parameters

The two-way ANOVA analysis demonstrated that the THC was altered significantly by the factors time ($F = 13.07$, $p = 0.001$), treatment ($F = 7.32$, $p = 0.000$) and treatment-time interaction ($F = 7.59$, $p = 0.000$). As showed in Fig. 3A, the pairwise comparison test revealed a significant decrease of THC values after 7 days of exposure to 1 µg/L of C6O4 or PFOA ($p < 0.001$). Both volume and diameter of haemocytes were affected by time ($F = 7.32$, $p = 0.010$ and $F = 7.59$, $p = 0.009$, respectively) and treatment ($F = 8.27$, $p = 0.000$ and $F = 7.06$; $p = 0.000$, respectively). In details, a significant increase in both volume and diameter of haemocytes was recorded after 7 days of exposure to 1 µg/L of C6O4 ($p < 0.001$) or 1 µg/L of PFOA ($p < 0.05$ for volume and $p < 0.01$ for diameter) (Fig. 3B, C).

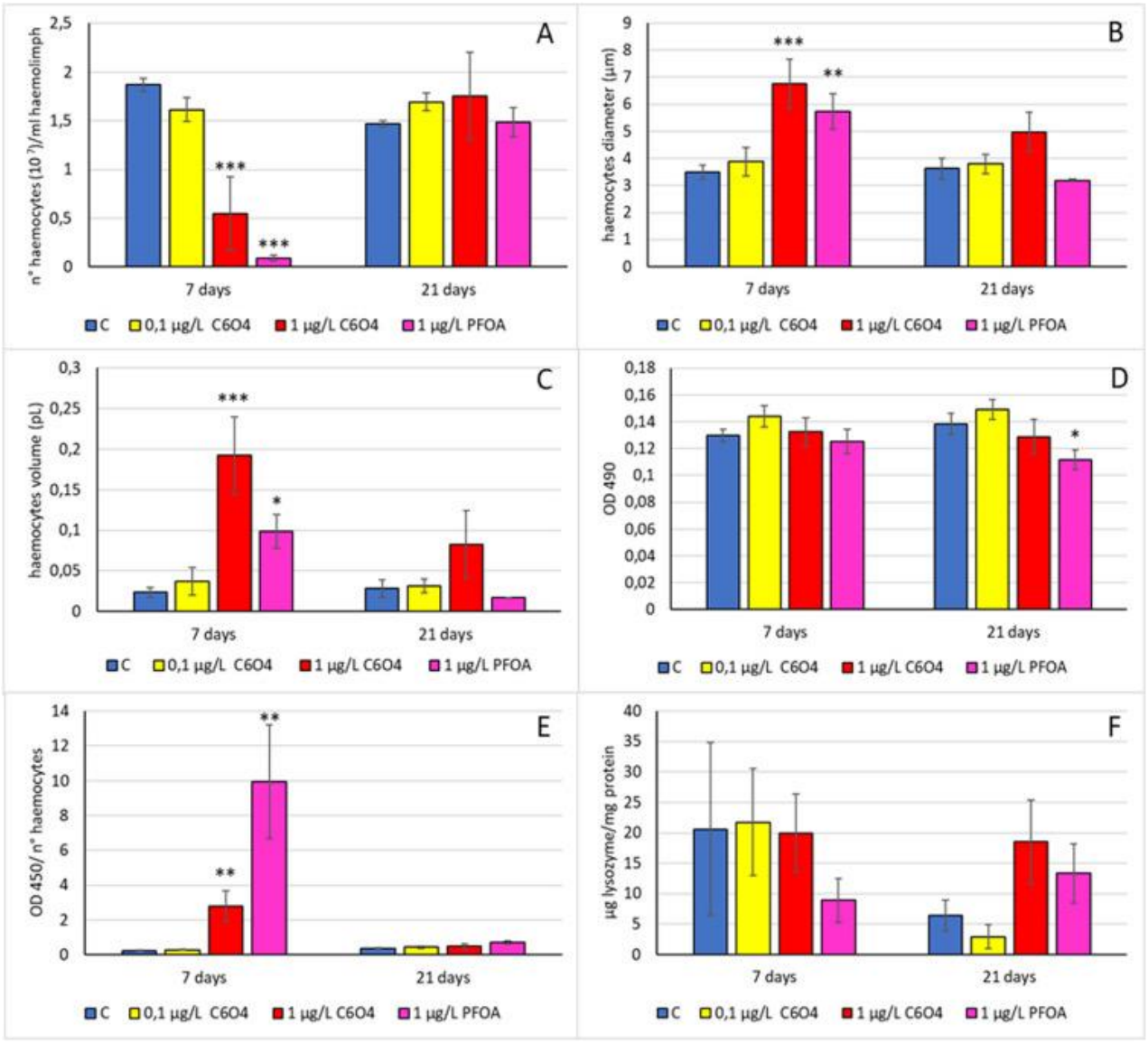

Figure 3. Total haemocyte count (THC), expressed as n° haemocytes (107)/mL haemolymph (A), haemocyte diameter, expressed in µm (B), haemocyte volume, expressed in pL (C), cell-free haemolymph lactate dehydrogenase activity, expressed as OD490 (D), haemocyte proliferation, expressed as OD450/n haemocytes (E), haemocyte lysate lysozyme activity, expressed as µg lysozyme/mg protein (F). The values are mean ± SE (n = 5). The asterisks indicate significant differences in comparison with control: *p < 0.05, **p < 0.01, ***p < 0.001.

Exposure to the compounds affected significantly ($F = 3.48$, $p = 0.027$) LDH activity. In detail, a significant decrease in enzyme activity was recorded after 21 days of exposure of clams to 1 µg/L of PFOA ($p < 0.01$) (Fig. 3D).

The factors time ($F = 11.02$, $p = 0.002$), treatment ($F = 7.75$, $p = 0.000$) and time-treatment interaction ($F = 6.82$, $p = 0.001$) significantly influenced haemocyte proliferation, and the pairwise comparison test revealed a significantly ($p < 0.01$) higher cell proliferation in bivalves exposed for 7 days at 1 µg/L of C6O4 or PFOA (Fig. 3E). All the factors considered did not affect lysozyme activity (Fig. 3F).

5.4.2 Gill and digestive gland parameters

In gills, SOD activity was affected significantly by treatment ($F = 3.02$, $p = 0.044$) and time-treatment interaction ($F = 3.60$, $p = 0.023$), with a significant increase after 7 days of exposure at the two C6O4 concentrations tested ($p < 0.05$) and at 1 µg/L of PFOA ($p < 0.001$) (Fig. 4A). Conversely, digestive gland SOD activity was affected by time only ($F = 9.99$, $p = 0.003$) (Fig. 4B).

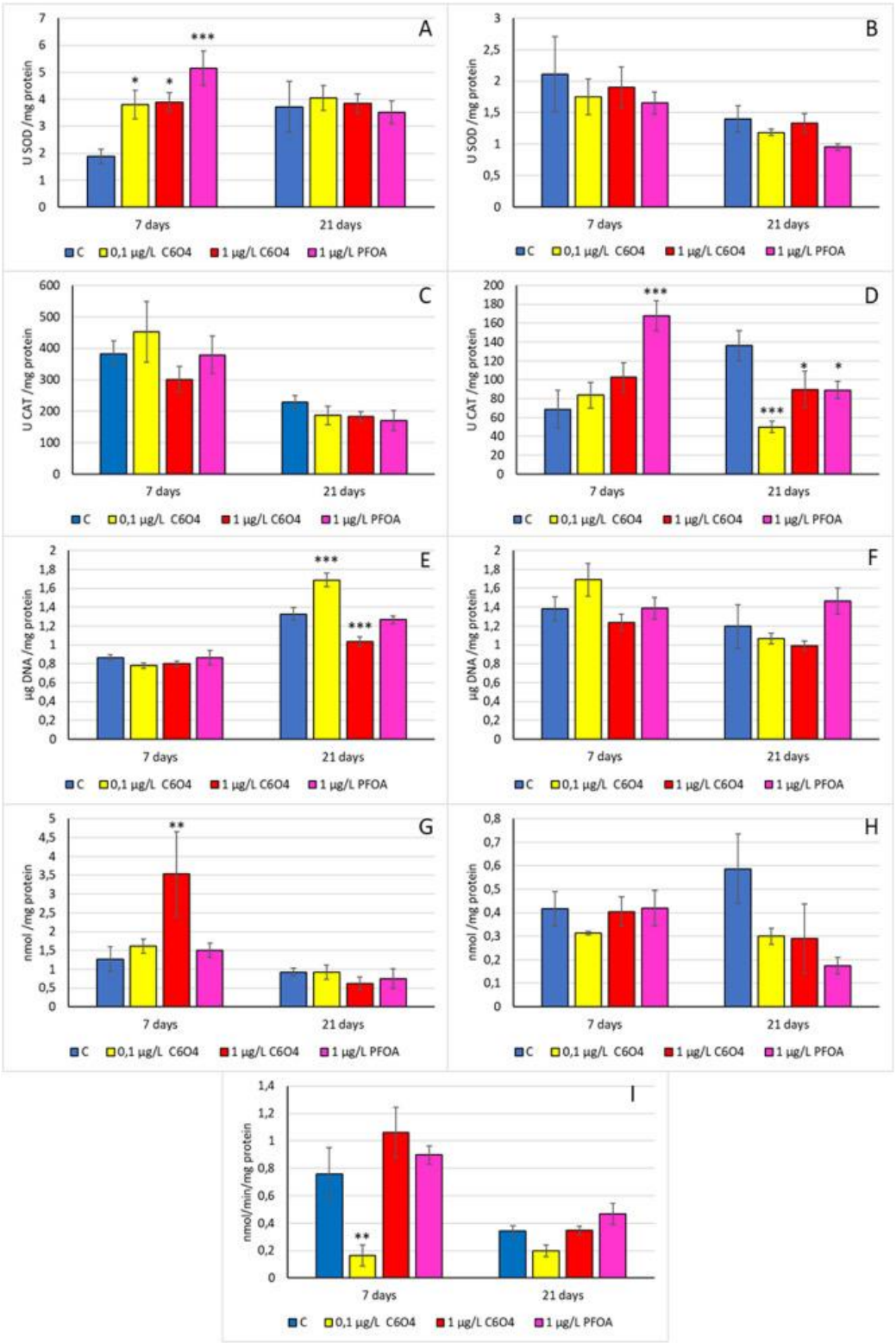

Figure 4. SOD activity, expressed as U SOD/mg protein, in gills (A) and digestive gland (B), CAT activity, expressed as U CAT/mg protein, in gills (C) and digestive gland (D), DNA strand breaks expressed as µg DNA/mg protein, in gills (E) and digestive glands (F), protein carbonyl content, expressed as nmol/mg protein, in gills (G) and digestive gland (H), AChE activity in gills, expressed as nmol/min/mg protein (I). The values are mean ± SE (n = 5). The asterisks indicate significant differences in comparison with control: *p < 0.05, **p < 0.01, ***p < 0.001.

D2.3.3.3-Caratterizzazione delle risposte nella vongola R. philippinarum a diversi inquinanti emergenti. Analisi chimiche molecolari (trascrittomiche), biochimiche e cellulari in vongole esposte a diversi contaminanti/miscele.

D2.3.3.4-Definizione dei meccanismi di azione di diversi inquinanti emergenti e dei possibili rischi per le attività produttive e l'ecosistema.

Contrary to what was observed for SOD activity, gill CAT activity was influenced significantly only by time ($F = 29.68$, $p = 0.000$), whereas treatment ($F = 5.68$, $p = 0.003$) and treatment-time interaction ($F = 8.19$, $p = 0.000$) significantly affected CAT activity in digestive gland. In that tissue, the pairwise comparison test revealed a significant increase in CAT activity after 7 days of exposure to 1 µg/L of PFOA ($p < 0.001$), while a significant decrease was recorded after 21 days of exposure to 0.1 µg/L of C6O4 ($p < 0.001$), 1 µg/L of C6O4 ($p < 0.05$) or 1 µg/L of PFOA ($p < 0.05$) (Fig. 4C, D).

GR gills activity was affected significantly only by time ($F = 377.87$; $p = 0.000$), while digestive gland GST and GR activities were not influenced by any considered factors (supplementary material, S2).

As for oxidative damage biomarkers, gill DNA strand breaks were affected significantly by the factors time ($F = 189.95$, $p = 0.000$), treatment ($F = 12,84$, $p = 0.000$) and treatment-time interaction ($F = 15.66$, $p = 0.000$), with a significant ($p < 0.001$) increase in animals exposed to 0.1 µg/L of C6O4 followed by a significant decrease at 1 µg/L of C6O4 ($p < 0.001$) after 3 weeks of exposure (Fig. 4E). In digestive gland, the DNA strand breaks resulted influenced significantly by time ($F = 4.69$, $p = 0.038$) and treatment-time interaction ($F = 3.53$, $p = 0.025$), with a significant increase in bivalves exposed for 21 days to PFOA, when compared to the related control (Fig. 4F).

PCC values in gills were affected significantly by time ($F = 13.82$, $p = 0.000$) and treatment-time interaction ($F = 3.430$, $p = 0.029$), with a significant ($p < 0.01$) increase at 1 µg/L C6O4 after 7 days of exposure, when compared to the related control (Fig. 4G). On the contrary, all the factors considered did not affect PCC values in digestive glands (Fig. 4H).

Lastly, gill AChE was significantly affected by time ($F = 25.65$, $p = 0.000$), treatment ($F = 10.37$, $p = 0.000$) and time-treatment interaction ($F = 4.24$, $p < 0.05$). Pairwise comparisons revealed a significant ($p < 0.01$) reduction of AChE in gills of clams exposed for 7 days to 0.1 µg/L of C6O4 (Fig. 4I). No significant alterations in enzyme activity were recorded after 21 days of exposure.

5.4.3 CCA analysis

A plot of the first two components of the CCA analysis are shown in the biplot in Fig. 5. The explained correlations are 99% and 98% for the first and the second canonical correlation, respectively. All the patterns are well separated, highlighting a strong effect of the experimental design on the measured parameters. At time 1 (= 7 days of exposure) the treatment 3 (= animals exposed to the highest concentration of C6O4) is very separated (different) from the other ones. Treatment 3 showed higher values of haemocyte diameter and volume and gill PCC, but lower THC values. A similar pattern can be seen at time 2 (= 21 days of exposure), with treatment 3 being separated from the other treatments, while the overall effects seem reduced. Furthermore, a clear time-dependent pattern of variation can be observed (time 1 on the right, time 2 on the left), with decreasing values of gill AChE, GR and CAT activities and increasing values of gill DNA strand breaks over time.

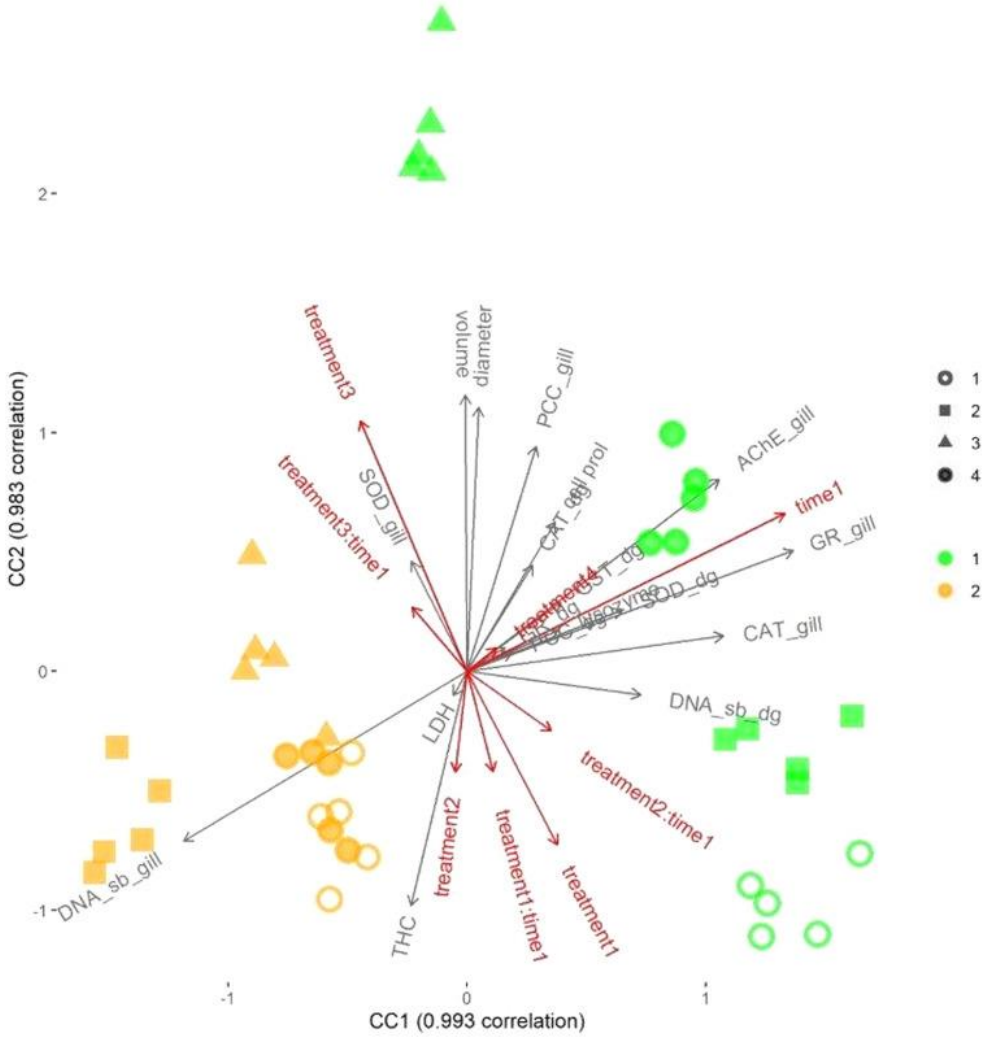

Figure 5. CCA biplot. Cellular and biochemical parameters measured in clams, as well as experimental concentrations were used for CCA analysis. Black symbols indicate experimental concentrations (1 =0 µg/L; 2 =0.1 µg/L C6O4; 3 =1 µg/L; 4 =1 µg/L PFOA), green symbol indicates time 1 (=7 days of exposure) and yellow symbol indicates time 2 (=21 days of exposure).

5.5 Discussion

5.5.1 Haemocyte parameters

It is well known that exposure to toxic substances can cause alterations of haemocyte parameters in molluscs (Matozzo and Gagné, 2016). In the present study, the effects of C6O4 and PFOA were investigated for the first time in haemocytes of *R. philippinarum*. Results demonstrated that the exposure to PFOA and C6O4 (at 1 µg/L) influenced haemocyte parameters, mostly after 7 days of exposure. We observed a reduction in THC values in haemolymph of clams exposed for 7 days, along with a significant increase of haemocyte volume and diameter. THC is one of the most used cell parameters to assess the effects of contaminants in bivalves at the cellular level (Matozzo and Gagné, 2016). The increase in THC values can be due to increased cell proliferation or movement of haemocytes from tissues to haemolymph, whereas a decrease in the THC values can be a consequence of cell death or an increased movement of haemocytes from haemolymph to

peripheral tissues (Pipe and Coles, 1995). In this study, the reduced number of circulating haemocytes in clams exposed for 7 days to C6O4 and PFOA could be due to either substances-mediated stressful condition or increased movement of haemocytes towards peripheral tissues to increase immunosurveillance. At 21 days, THC values restored in treated clams, suggesting a recovery capability of bivalves. Regarding previous studies, it has been demonstrated that exposure for 21 days of the Chinese mitten-handed crab *Eriocheir sinensis* to PFOS (0.01–10 mg/L) caused a significant reduction in THC values, starting from the concentration of 0.1 mg/L (Zhang et al., 2015).

Overall, C6O4 and PFOA at a concentration of 1 µg/L seemed to act in a very similar way on clam haemocytes, determining a decrease in THC and an increase in size after 7 days of exposure. The increase in haemocyte size can be a consequence of alteration in cell membrane permeability, which leads to increased cell volume. Unfortunately, no studies are available in the literature concerning the effects of PFASs on cell volume and diameter. Consequently, it is difficult for us to propose a plausible explanation for these results. In our previous study, we demonstrated that haemocytes from *R. philippinarum* can divide in haemolymph (Matozzo et al., 2008). In this study, the XTT assay revealed a significant increase of haemocyte proliferation in clams exposed for 7 days to PFOA and C6O4. In a similar way for the two compounds, cell proliferation returned to control levels after 21 days of exposure. We hypothesised that increased cell proliferation in C6O4- and PFOA-treated animals was an attempt of clams to re-establish cell number that dropped after 7 days of exposure. An increase in cell proliferation was also observed in carp (*Cyprinus carpio*) hepatocytes following in vivo exposure for 56 days to PFOA (200 ng/L to 2 mg/L), even if at the highest concentration tested only (Giari et al., 2016).

In the present study, no significant reductions in haemocyte membrane stability (as indicated by the results of LDH assay) was observed in clams exposed to PFOA and C6O4. Similarly, in a recent study, Coperchini et al. (2021) demonstrated that in vitro exposure of human thyroid cells to six C6O4 concentrations (0, 0.01, 0.1, 1, 10 and 100 ng/mL) did not cause any alteration of cell viability, ROS production and cell proliferation. On the contrary, Tang et al. (2018) observed a cytotoxic effect after 12 h of in vitro exposure of lymphocytes from the freshwater *Carassius auratus* to PFOA (10 and 100 µg/L), followed by an induction of autophagy pathway genes.

Lysozyme is an important hydrolytic enzyme that can be released by haemocytes into haemolymph during phagocytosis to counteract non-self materials. In the present study, no significant alterations of lysozyme activity were observed in haemocyte lysate of clams exposed to C6O4 and PFOA, suggesting that lysozyme is not a target for the two compounds. Conversely, the enzymatic activity decreased in the liver of the of zebrafish *Danio rerio* following a 21-day exposure to 0.04 mg/L of PFOS (Guo et al., 2019) and in cell-free haemolymph of *E. sinensis* following exposure for 21 days to PFOS (0.01–10 mg/L) (Zhang et al., 2015).

5.5.2 Gill and digestive gland parameters

One of the possible consequences of exposure of marine organisms to environmental contaminants is oxidative stress, a condition provoked by and imbalance between the production of reactive oxygen species (ROS) and antioxidant defences. Recent studies have shown the effects of different PFASs on oxidative stress biomarkers in marine organisms (Wu et al., 2019, Miranda et al., 2020). To assess the capability of C6O4 and PFOA to induce oxidative stress in clams, as well as oxidative damage to proteins and DNA, several biomarkers were evaluated in this study. As it is well known, SOD catalyses the dismutation of superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2). In this study, gill SOD activity was affected by exposure of clams to contaminants. Indeed, after 7 days of exposure enzyme activity increased significantly in C6O4- and PFOA-treated groups, compared to control ones. After 21 days of exposure, SOD activity values resulted to be like those of controls, with no significant differences between the experimental conditions. Unlike in gills, SOD activity in the

digestive gland was significantly affected by exposure duration only. In our opinion, the different response of gills and digestive gland to contaminant exposure could be due to their location in animals. Indeed, gills are the first barrier between the external and internal environment of bivalves and as such must activate antioxidant defence mechanisms against oxidative stress. On the other hand, digestive gland has a different position and role in bivalves. Therefore, it is plausible that defences against oxidative stress activate later than gills. Previous studies have shown a different pattern of variation of antioxidant enzymes following exposure of organisms to various concentrations of contaminants. For example, SOD activity increased at the lower concentrations (0–100 µg/L), while it decreased at the highest concentrations (100–10,000 µg/L) in the green mussel *Perna viridis* exposed for 7 days to various perfluorinated compounds, such as PFOS, PFOA, PFNA, PFDA (Liu et al., 2014a). Similarly, in the freshwater mollusc *Unio ravoisieri* exposed for 7 days to PFOS (2–10 mg/L) gill and digestive gland SOD activity increased in animals treated with 2 and 6 mg/L, while it decreased in molluscs exposed to 10 mg/L (Amraoui et al., 2018). Likewise, an increase in SOD activity was observed in the amphipod *Gammarus insensitives* treated for 4 days with 1 mg/L of PFOS, followed by a decrease in those exposed to 1.6 and 3.1 mg/L (Touaylia et al., 2019). Conversely, in the liver of *D. rerio* SOD activity increased following exposure for 96 h to 200 µg/L of PFOS, whereas no alterations were observed in gills (Li et al., 2017). Similarly, in *C. auratus*, no SOD activity alterations have been detected in liver exposed to 1.21 and 12.1 µmol/L of PFOA for 4 days (Feng et al., 2015). In other studies, a reduction in SOD activity has been demonstrated following exposure to PFASs. For example, SOD activity decreased in *E. sinensis* exposed for 21 days to highest concentrations of PFOS (till to 10 mg/L) (Zhang et al., 2015) and in the freshwater cladoceran *Daphnia magna* exposed for 7 days to PFOS or PFNA (0.008–5 mg/L), just at the concentration of 0.2 mg/L for both the compounds (Lu et al., 2015).

CAT catalyses the conversion of hydrogen peroxide (H_2O_2) to water and molecular oxygen and is generally found within peroxisomes. In this study, differences in CAT activity were observed between gills and digestive gland. Gill CAT activity was significantly affected by exposure duration only, whereas digestive gland CAT was influenced significantly by both treatment and time-treatment interaction. After 7 days of exposure, a significant increase in enzymatic activity was detected in digestive gland from animals treated with PFOA. After 21 days of exposure, a significant decrease in CAT activity was observed in digestive gland of animals exposed to all the concentrations tested. In previous studies, a reduction of CAT activity was detected in the liver of *Oryzias latipes* exposed for 7 days to PFOA (10, 50, 100 mg/L) (Yang, 2010) and in *D. magna* exposed for 7 days to PFOS or PFNA (0.008–5 mg/L), just at a concentration of 0.04 mg/L for both the compounds (Lu et al., 2015). Conversely, increased CAT activity was recorded both in digestive gland and in gills from *U. ravoisieri* exposed to PFOS (2–10 mg / L) (Amraoui et al., 2018). In the Australian rainbowfish *Melanotaenia fluviatilis* exposed for 24 days to PFOA (0.01, 0.1, 1 and 10 mg/L), an increase in CAT activity was recorded in the gills of animals exposed to 0.1 mg/L, whereas a reduction was found in the liver of fish treated with the two highest concentrations tested (Miranda et al., 2020). Increases in CAT activity were also observed in *P. viridis* following exposure for 7 days to PFOS, PFOA, PFNA, PFDA (0–10,000 µg/L), showing an increase in enzyme activity up to a concentration of 100 µg/L (Liu et al., 2014b). In addition, a significant increase in CAT activity was observed in hepatocytes of *O. niloticus* exposed for 24 h to PFOS or PFOA (1, 5, 15 and 30 mg/L), but at the highest concentration tested only (Liu et al., 2007).

GR is an important enzyme that plays a key role in antioxidant defences, as it converts oxidised glutathione (GSSG) to reduced glutathione (GSH), thus replenishing functionally active GSH reserves. Although no significant effects of exposure to C6O4 and PFOA were observed in our study, a different pattern of variation of GR activity was recorded between gills and digestive gland of *R. philippinarum*. GR activity significantly decreased over time in gills of clams exposed to all the experimental conditions (including controls), whereas it did not change in digestive gland throughout the experiments. Overall, at the end of exposure (21 days), GR activity in gills resulted about five times lower than in digestive gland. An increase in GR activity was also

observed in hepatocytes from *O. niloticus* exposed for 24 h to PFOS or PFOA (1, 5, 15 and 30 mg/L), particularly at the 5 mg/L of PFOA and at 30 mg/L of PFOS (Liu et al., 2007).

GST is a detoxifying enzyme of phase II of biotransformation that catalyses the conjugation of glutathione with toxic substances, to make them less reactive and more easily eliminated by organisms. Therefore, GST is indirectly involved in the antioxidant defence since it affects the availability of glutathione. In this study, digestive gland GST activity was not significantly influenced by either the treatment or the exposure time. On the contrary, alterations in GST activity were observed in the gills of *M. fluviatilis* exposed for 24 days to PFOA (0.01, 0.1, 1 and 10 mg/L), in which an increased enzyme activity was recorded at the two highest concentrations tested, while a decreased activity was found in the liver of animals treated with the highest concentration tested (Miranda et al., 2020). Increased GST activity was also observed in *Dugesia japonica* exposed for 10 days to PFOA (0.5, 5, 10, 20 mg/L) (Yuan et al., 2017), and in digestive gland of *M. galloprovincialis* exposed for 21 days to PFOS (2–10 mg/L) (Gülsever and Parlak, 2018). Conversely, inhibition of GST activity was detected in hepatocytes of *O. niloticus* exposed for 24 h to PFOS or PFOA (1, 5, 15 and 30 mg/L), even if only at the highest concentrations tested of both the compounds (Liu et al., 2007).

Protein carbonylation is one of the possible consequences of oxidative stress and PCC determination is a useful biomarker of oxidative damage to proteins (Dalle Donne et al., 2003). In our study, gill PCC was significantly affected by exposure time and time-treatment interaction. In particular, a significant increase in PCC was observed in animals treated for 7 days with 1 µg/L C6O4, with respect to the related control. Conversely, PCC in digestive gland was affected by exposure time only. Results obtained suggested that gills are more vulnerable than digestive gland to oxidative damage, at least after short-term exposure of clams to C6O4. To the best of our knowledge, no literature data on the effects of PFASs on PCC in aquatic organisms are available. Just for a comparison on the effects of other contaminants to mollusc proteins, PCC was shown to increase in the gills and digestive gland of *R. philippinarum* following exposure to titanium dioxide nanoparticles (Marisa et al., 2018), in the scallop *Chlamys farreri* exposed to chrysene (Xiu et al., 2016) and in the freshwater mussel *Dreissena polymorpha* exposed to amphetamine (Parolini et al., 2016).

Oxidative damage to DNA can occur because of oxidative stress following exposure of organisms to contaminants. In our study, DNA strand breaks in gills was significantly affected by both exposure time and treatment, with a significant increase after 21 days of exposure of clams to C6O4 (0.1 µg/L) and a decrease in PFOA-exposed animals. Conversely, no significant variations were recorded in clam digestive gland, highlighting once again the greater vulnerability of gills than digestive gland. Although a significant variation in DNA strand breaks was observed after 21 days in gills from clams exposed to C6O4, two different patterns of variation were observed, as the values increased at the lowest concentration and decreased at the highest concentration of C6O4. In this regard, we speculated that exposure to the highest concentration of C6O4 was able to activate DNA repair enzymatic systems. Increases in DNA strand breaks was observed in the planarians *Dugesia japonica* exposed for 4 days to 15 mg/L of PFOA (Zhang et al., 2020), in *P. viridis* exposed for 7 days to PFOS or PFOA (0.1–1000 µg/L) (Liu et al., 2014b), and in *C. carpio* after exposure for 4 days to PFOS (50–50,000 µg/L) starting from the nominal concentration of 5000 µg/L, while no genotoxic effects were observed in PFOA-treated fish (Kim et al., 2010).

AChE converts acetylcholine to choline and acetic acid at the synaptic junctions, playing a key role in the transmission of the nerve signals. In our study, AChE activity was significantly influenced by treatment, exposure time and treatment-time interaction, with a significant decrease in animals exposed for 7 days to the lowest concentration of C6O4. Results obtained suggested a neurotoxic potential of C6O4 after short-term exposure and a recovery capability of clams after more prolonged exposure. Previous studies demonstrated that PFASs can reduce markedly AChE activity in various species, as observed in *D. japonica* exposed for 10 days to 15 mg/L of PFOA (Zhang et al., 2020), in *D. magna* exposed for 7 days to PFOS or PFNA (0.008–5 mg/L) (Lu et al., 2015), and in the brain of *C. auratus* after exposure for 7 days to PFOA (0.2–

25,000 µg/L) (Dong et al., 2019). Conversely, no significant alterations in AChE activity were found in *C. carpio* brain samples after exposure for 4 days to PFOS or PFOA (50–50,000 µg/L) (Kim et al., 2010), in *U. ravoisieri* exposed for 7 days to 2 and 10 mg/L of PFOS (Amraoui et al., 2018) and in *G. insensibilis* exposed for 4 days to PFOS (1–3.1 mg/L) (Touaylia et al., 2019).

Interestingly, we observed that the foot of at least five clams exposed to the lowest concentration of C6O4 (0.1 µg/L) was cut off, clams being unable to withdraw it before shell closure. Such observation, along with the reduction of AChE in clams from the same experimental group, supports the hypothesis of a possible neurotoxic effect of the contaminant. Likewise, both syphons and foot of clams were cut-off following exposure to 4-nonylphenol (Matozzo et al., 2003). In any case, narcotic effects of C6O4 to clams cannot be excluded. In this regard, further investigations are needed to evaluate deeply the narcotic and/or neurotoxic effects of C6O4 to aquatic species.

5.6 Conclusions

Results of the present study demonstrated that C6O4 and PFOA can affect biomarker responses in *R. philippinarum*. Of the biomarkers measured, haemocyte parameters resulted influenced by exposure to the two compounds similarly to biochemical ones. Exposure to contaminants affected THC, haemocyte volume and diameter, and cell proliferation. However, values of these parameters generally returned to control values after 21 days of exposure, indicating a transient effect of the contaminants. Interestingly, biomarkers measured in clam gills were more affected by contaminant exposure than those evaluated in digestive gland. In particular, C6O4 induce more DNA alterations than PFOA, with an increase of DNA damage at the lower concentration and a reduction of DNA damage at the higher concentration tested. Lastly, evidence of reduction in AChE activity in gills of clams exposed to 0.1 µg/L of C6O4, along with the observation of some cases of clams with foot cut off in the same experimental group, are noteworthy and suggest that other studies are necessary to investigate more fully the effects of such compounds in clams, as well as in other aquatic invertebrate species.

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6. Response of the Mediterranean mussels *M. galloprovincialis* to Venice lagoon concentrations of the fragrance Amyl salicylate

6.1 Introduction

Fragrances are "chemical mixtures obtained by natural aromatic raw and/or synthetic materials that smell of characteristic odour" as defined by the International Fragrance Association (IFRA). They are used as ingredient in several daily life Personal Care Products (PCPs), such as preparation of perfumes, cosmetics, toiletries et cetera (Bauer et al., 1997). Fragrances, and PCPs more in general, are widely produced all over the world (Liu and Wong, 2013) and following their extensive application, they and their metabolic conjugates are discharged in wastewaters (Patel et al., 2009). Going through sewage treatment plants (STPs) they may end up in marine environment (Díaz-Cruz et al., 2009), assuming the role of potential threat for biota and abiotic habitats mainly due to their persistence and/or their ceaseless inputs (Casatta et al., 2015; Chase et al., 2012). Nevertheless, this class of pollutants is not currently included in routine monitoring programs of European Union although they may present a significant toxicological risk for the environment and human health (Patel et al., 2009). Overall considered, scientific community has classified fragrances as Contaminants of Emerging Concern (CECs) (Tang et al., 2017), implying the need of further studies on their eco – and human toxicological risk, even with the aim to provide scientific bases addressed to regulations of European legislation filling the critical data gap of potential environmental impacts of these emerging contaminants (Chase et al., 2012).

Among aqueous systems affected by these chemical compounds, even fragile ecosystems as lagoons seem not to be "fragrances-free" (Casatta et al., 2015; Vecchiato et al., 2017, 2016). The Lagoon of Venice, for instance, a vulnerable fragile and complex ecosystem with relevant ecological and commercial importance, represents a significant case regarding fragrances contamination. Vecchiato et al (2016) reported the presence of several fragrances in water surface of the Venice lagoon, highlighting salicylates compounds as among the most abundant and widespread components (Vecchiato et al., 2016).

Amyl salicylate (C12H16O3), (IUPAC name: pentyl 2-hydroxybenzoate) (CAS No 2050-08-0), is one of the members of the salicylate family, among methyl, butyl, hexyl, benzyl and 3-hexenyl salicylates, widely used in perfumery and in other personal care products because of its organoleptic properties and the low cost (under $5/kg) (Gaudin, 2014). Its use worldwide increased along the last two decades, amounting around 100–1000 metric tonnes per annum (Belsito et al., 2007; Lapczynski et al., 2007). Because of the extensive usage, the fate of this compound might be the same of that of other fragrances and PCPs. Even if studies regarding the environmental occurrence of Amyl salicylate are slightly carried out, its presence in aquatic systems has been reported. Vecchiato and colleagues found Amyl salicylate being one of the main fragrances detected in Antarctic waters ranging at 4.2–29 ng L-1, but reaching concentrations up to 6 µg L-1 in urban environments as in the innermost canals of Venice (Vecchiato et al., 2017, 2016). Moreover, detectable levels between 7 and 50 ng/L of AS were found in Elbrus (Caucasus) in all investigated points of the survey (Vecchiato et al., 2020). Despite Amyl salicylate is considered one of the most stable and long-lasting fragrances in sea water (Vecchiato et al., 2016) that is arousing emerging concern, only few studies has yet explored the potential accumulation and effects of this compound for aquatic fauna to date. Amyl Salicylate was recently recognized to affect the larval development of the copepod *Acartia tonsa* with EC50 = 131 ng L−1 (Picone et al., 2021) where a positive correlation between the concentration and the effects was detected confirming the potential risk of this CEC in water environment (Picone et al., 2021). Moreover Fabrello et al. (2021) tested the effects of a mixture of CECs, including Amyl salicylate in *Mytilus galloprovincialis* highlighting negative consequences at biochemical and cellular level, such as in Total Haemocytes Count (THC), haemocytes proliferation, LDH and in few antioxidant enzymes activity. However, still little

information on the hazard of this single fragrance is available to date and future researches should characterise its effects and fate following the biota and environmental interactions.

According to the classification of the Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (the "CLP Regulation") that replaced the Directive 67/548/EEC, Amyl salicylate is considered dangerous for the environment and in particular very toxic to aquatic life with long lasting effects to aquatic organisms by ECHA in REACH registrations (ECHA, 2020).

Overall, the present experiment was set to investigate for the first time effects at cellular, biochemical and molecular levels in Mediterranean mussels (*M. galloprovincialis*), an ecological and economic important edible bivalve species and widely used for ecotoxicological studies, of exposure to realistic concentrations of Amyl salicylate. To reach such a goal, a set of biomarkers and gene expression profiles (RNA-seq) were studied. Furthermore, because microbiota play important roles in molluscs, as in nutrient assimilation, homeostasis, immunological regulation, maintenance of pH and redox potential of the gut (Aceves et al., 2018; Musella et al., 2020) and the protection against pathogens and environmental stressors (Milan et al., 2018), structure and diversity of microbial communities in the digestive gland of mussels was studied through 16S rRNA Amplicon Sequencing technique in order to understand mussels' health status following the Amyl salicylate exposure.

6.2 Material and methods

6.2.1 Experimental design

270 adults of Mediterranean mussels (*Mytilus galloprovincialis*) (53.8±5.1 mm) used in the present experiment were collected in an unpolluted bivalve farming area in the south of the Venice Lagoon (Italy). Samples were left to acclimate with aerated seawater (salinity of 35 ± 1, temperature of 18 ± 0.5 °C) for one week and fed daily with *Isochrysis galbana*. A total of 45 animals were then placed in glass aquarium containing 30 L of clean water, previously collected in the Venice Lagoon and transported to the laboratory for the experimental manipulation. Water was renewed every two days during the experiment. In this experiment, we used Amyl salicylate (AS) (CAS no.: 2050-08-0) recently detected as the fragrance ingredient with the highest concentrations in waters of an area in the Venice Lagoon (0.3 µg/L; unpublished data). Three experimental groups performed in duplicate were design: mussels were exposed to 0 µg/L (control group; CTRL), 0.3 µg/L Amyl salicylate (lower concentration; AS_L) and 3 µg/L Amyl salicylate (high concentration; AS_H) for 7 and 14 days. Accordingly, AS concentrations used in the present study were chosen basing on realistic environmental detections in the Venice Lagoon.

6.2.2 Chemical analysis

Concentration of Amyl salicylate was measured in water samples of each experimental group. Water was collected approximately 15 min after chemical application (t = 0), after 24h and after 48 h (before water renewal and the second application).

Water samples (0.05 L) were extracted onto Oasis HLB cartridges 6cc (200 mg), Waters, Milford, MA, (USA), previously conditioned with *n*-hexane, dichloromethane and ultrapure water (10 mL each) using phenanthrene ^{13}C as internal standard. Samples were eluted with 1 mL of toluene, followed by 15 mL of dichloromethane and 10 mL of *n*-hexane. Eluates were dried with Na_2SO_4 and concentrated to 100 µL under a gentle nitrogen flow at 23 °C (Turbovap II®, Caliper Life Science, Hopkinton, MA, USA). Mussels of each condition used for chemical analyses were sampled at 7th and 14th day of exposure. The pools of the total

mussel tissues (5 animals each experimental group) were homogenized using Ultra-Turrax (IKA) extracted by means of a QuEChERS method. About 3.7 g of wet sample were weighted into a 50 mL PP centrifuge tube and spiked with phenanthrene ^{13}C as internal standard, together with 7 mL of UPW and 10 mL of acetonitrile. After 1 min of vortex, 6 g of magnesium sulfate, 1.5 g of sodium chloride, 0.75 g of sodium citrate dibasic sesquihydrate, and 1.5 g of sodium citrate tribasic dihydrate were added for phase-separation adjustment. Samples were vortexed for 1 min and centrifuged for 5 min at 3000 RPM. Supernatants were transferred to another centrifuge tube contaning 825 mg of Supelclean™ PSA (Primary-Secondary Amine), 2.5 g of magnesium sulfate, 1 g of Na_2SO_4 and 825 mg of Discovery® DSC-18, vortexed for 1 min and centrifuged for 5 min at 3000 RPM. Samples were concentrated to 100 µL, with solvent exchange with dichloromethane. Instrumental analyses were performed by GC-MS/MS (Trace 1310 - TSQ 9000 Thermo Fisher) monitoring the transition 120>92 (CE 10V) for quantification and 138>92 (CE 15V) and 138>120 (CE 5V) for confirmation. Analyses were performed onto a 60-m HP-5MS column (0.25 mm I.D., 0.25 µm; Agilent Technologies, Avondale, USA), with He at 1.2 ml min^{-1} and oven starting at 120°C (1 min), increasing to 180°C at 25°C min^{-1}, to 250°C at 10°C min^{-1} and to 310°C at 20°C min^{-1} (1 min).

6.2.3 Biomarkers

Haemolymph was collected from the anterior adductor muscle by a 1-mL plastic syringe and stored in Eppendorf tubes at 4 °C. At each sampling time, 5 pools of haemolymph (from four mussels each) from each experimental condition were prepared. A volume of pooled haemolymph was immediately used to measure total haemocyte count (THC), haemocyte diameter and volume, lactate dehydrogenase (LDH) activity and haemocyte proliferation (XTT). The remaining haemolymph was then centrifuged at 780 x g for 10 min, the supernatant was discharged, while the pellets (=haemocytes) were resuspended in distilled water to obtain haemocyte lysate (HL) samples. Briefly, haemocytes resuspended in distilled water were vortexed for 30 secs and centrifuged at 780 x g for 10 min at room temperature. The supernatant, corresponding to HL, was then collected, frozen in liquid nitrogen and stored at -80°C until analyses (lysozyme activity assay).

After haemolymph collection, gills and digestive gland were excised, pooled (five pools of four mussels each per each experimental condition) and divided in aliquots. Aliquots were then frozen in liquid nitrogen and stored at -80°C until analyses.

Total haemocytes count (THC), as well as haemocyte diameter and volume, were determined using a Scepter™ 2.0 Automated Cell Counter (Millipore, FL, USA). Briefly, 20 µL of haemolymph were added to 2 mL of Coulter Isoton II diluent. The THC was expressed as the number of haemocytes (10^7)/mL of haemolymph, while haemocyte diameter and volume were expressed in µm and picolitres (pL), respectively.

Haemocyte proliferation was evaluated using the *Cell proliferation* Kit II, Roche (a commercial kit). Briefly, a volume of 200 µL of the mixture provided by the kit was added to 400 µL of pooled haemolymph and incubated for 4 hours in a dark humidified chamber. The absorbance at 450 nm was then recorded using a Beckman 730 spectrophotometer. The results were normalised to THC values of each experimental groups and expressed as optical density (OD) at 450 nm.

The commercial *Cytotoxicity Detection* Kit, (Roche) was used to measure lactate dehydrogenase activity (LDH) in cell-free haemolymph (CFH). Pooled haemolymph (500 µL) from each experimental condition was centrifuged at 780 x g for 10 min, and the supernatant (=CFH) was then collected for the assay following the manufacturer's instructions. The results were expressed as optical density (OD) at 490 nm.

Lysozyme activity was measured in haemocyte lysate (HL) from pooled haemolymph (500 µL). HL was obtained as described above. In details, 50 µL of HL was added to 950 µL of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma) in 66 mM phosphate buffer (pH 6.2), and the decrease in absorbance

(A/min) was continuously recorded at 450 nm for 3 min at room temperature. The results were expressed as µg lysozyme/mg of protein. Protein concentrations in the HL were quantified accordingly to Bradford (1976).

Gills and digestive gland samples were homogenised at 4 °C with an Ultra-Turrax homogeniser (model T8 basic, IKA) in four volumes of 10 mM Tris-HCl buffer, pH 7.5, containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA and protease inhibitor cocktail (1:10 v/v) (Sigma-Aldrich), and centrifuged at 12,000 g for 30 min at 4 °C. Supernatants (SN) were collected for analyses. The protein concentration in SN samples was quantified according to Bradford (1976).

Total superoxide dismutase activity (SOD) was measured in both gills and digestive gland in triplicate using the xanthine oxidase/cytochrome c method proposed by Crapo et al. (1978). Enzyme activity was expressed as U/mg protein, one unit of SOD being defined as the amount of sample causing 50% inhibition under the assay conditions.

Catalase activity (CAT) was measured in gill and digestive gland SN in triplicate following the method proposed by Aebi (1984). The enzyme activity in a volume of 30 µL of tissue SN were measured at 240 nm and expressed as U/mg protein; one unit of CAT was defined as the amount of enzyme that catalysed the dismutation of 1 µmol of H_2O_2/min.

Glutathione reductase (GR) activity was evaluated according to Smith et al. (1988), by measuring the (5-thio (2-nitrobenzoic acid)) TNB production at 412 nm. The enzyme activity was expressed as U/mg protein.

Glutathione S-transferase (GST) activity was measured in digestive gland SN according to the method described in Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates. GST activity was expressed as nmol/min/mg protein.

Acetylcholinesterase and butyrylcholinesterase activity (AChE and BChE, respectively) were evaluated in gill SN (25 µL) using the Ellman method (Ellman et al. 1961), following the colorimetric reaction between acetylthiocholine or butyrylthiocholine and the reagent dithiobisnitrobenzoate. Changes in absorbance were then recorded at 405 nm for 5 min on a microplate reader at room temperature. The results were expressed as nmol/min/mg of protein.

6.2.4 Molecular analysis

RNA was extracted from a pool of 4 digestive glands of animals of each experimental group (5 pools each condition) using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and with additional DNAse treatment (Qiagen). RNA concentration and integrity of each pool were checked using Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, United States). Extracted RNA from each pool was used for both gene expression (RNA-Seq) and microbiota analyses (16S).

Regarding gene expression analyses, libraries were constructed using 3'QuantSeq kit (Lexogen) and the pools were then sequenced on NextSeq500 Illumina (CRIBI; University of Padova) with a single-end (75 bp) setup.

For microbiota analyses, RNA was retro-transcribed to cDNA using the Superscript IV kit (Invitrogen, Life Technologies, Monza, Italy), then amplified including both reverse and forward primers that specifically target the V3–V4 gene region of the bacterial 16S rRNA. Sequencing was performed through 16S rRNA amplicon sequencing technique, as described by Milan et al., 2018, by BMR Genomic (Padua, Italy).

Raw sequencing data were analysed using QIIME2 (Quantitative insights into microbial ecology 2; Bolyen et al., 2019). Reads were trimmed, filtered, and merged with cutadapt and DADA2 respectively to exclude primer sequences and, based on the nucleotide assignment, reads with quality lower than 20 (Phred Score).

Features were aligned using MAFFT software (Katoh and Standley, 2013). After the quality-filter step, chimeric amplified fragments were removed and sequences merged to a total of 1.519.962 reads. Merged reads were classified with Python library scikit-learn and taxa assignment was performed using a pre-trained SILVA-database (Yilmaz et al., 2014). Final feature table and relative taxonomy were used for pairwise comparison (treated versus relative control group) carried out by CALYPSO (version 8.84). In detail, microbial characterization was studied through i) Principal coordinates analysis (PCoA) at species level, ii) the investigation of Evenness and Simpson's index at species level and iii) one-way ANOVA to evaluate changes in microbial composition at species and genus levels.

6.2.5 Chemical analyses

Chemical analyses of water used for mussels exposures showed background levels of Amyl Salicylate ranging between 2.6 ng L^{-1} and 12 ng L^{-1} in control samples. After the addition of Amyl Salicylate, 49-85 ng L^{-1} and 276-565 ng L^{-1} were detected in AS_L and AS_H tanks, respectively. However AS concentrations dropped to levels comparable to control tanks (3.3-17 ng L^{-1}) after 24h the addiction of AS. Concerning bioaccumulation, AS concentrations in CTRL mussels ranged between 2.2 ng g^{-1} and 2.3 ng g^{-1}, indicating previous exposures in Venice lagoon. Mussels exposed to 0.3 µg L^{-1}, showed AS concentrations of 2.9 ng g^{-1} and 3.2 ng g^{-1}, at 7 and 14 days respectively. As expected, the highest bioaccumulation were detected in individuals exposed to 3 µg L^{-1}, resulting in 6.9 ng g^{-1} and 5.9 ng g^{-1} after 7 and 14 days, respectively. Bioconcentration Factor (BCF) calculated were equal to 11 and 2 (14 days) in AS_L and AS_H, respectively.

6.2.6 Haemolymph, gill and digestive gland parameters

The two-way ANOVA analysis revealed a significant effect of the factors treatment ($F=30.14$, $p=0.000$) on THC values of mussels. In details, the post hoc test revealed a significant ($p<0.001$) decrease in THC after 7 days of exposure to AS_H (Fig. 1). Exposure of mussels to AS affected significantly both the volume and diameter of haemocytes ($F=4.46$, $p=0.022$ and $F=6.33$, $p=0.006$, respectively), with a significant ($p<0.01$) increase in both volume and diameter of haemocytes after 7 days of exposure to AS_H (Fig. 1B, 1C).

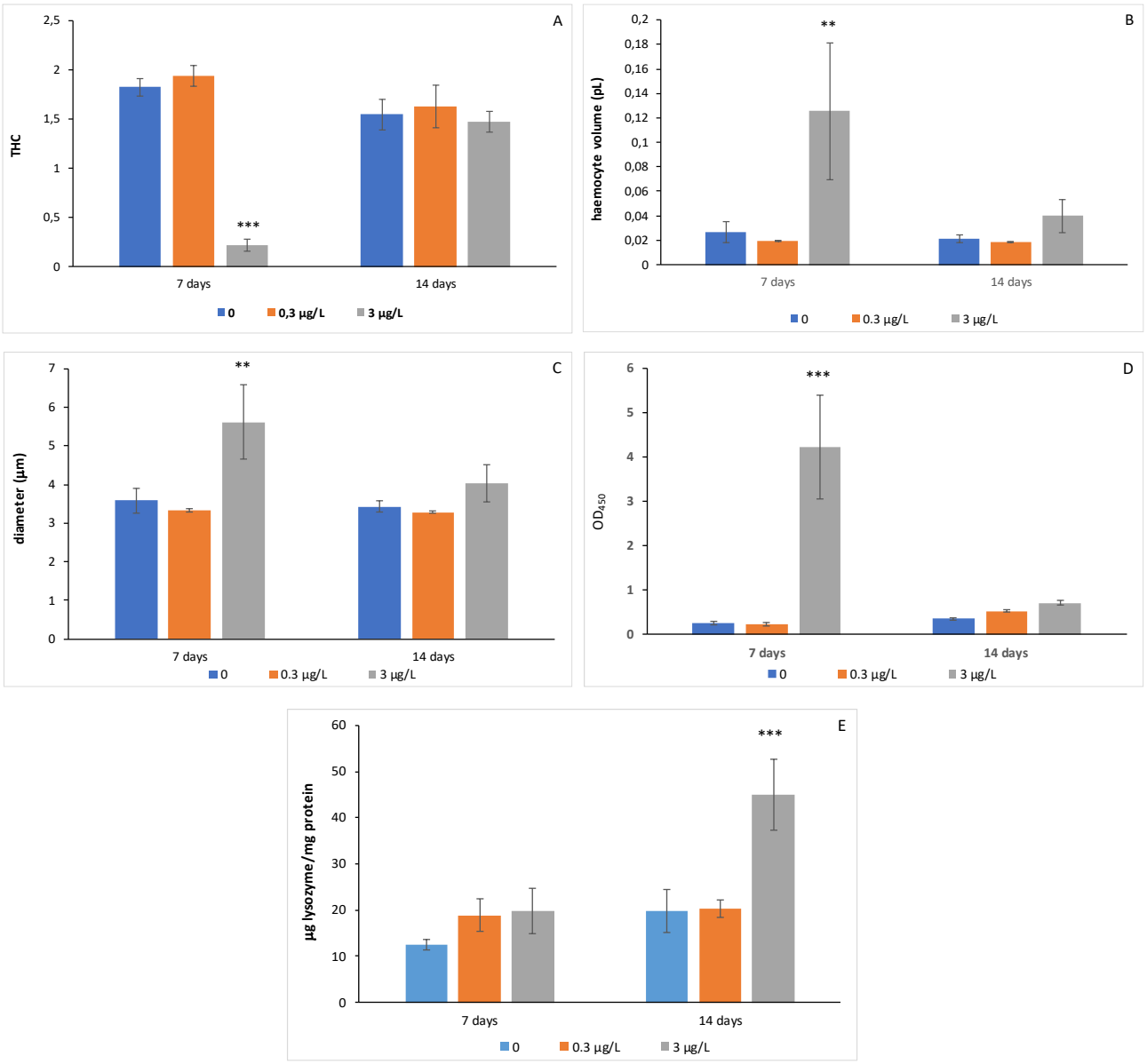

Figure 1. Total hemocyte count (THC), haemocytes volume, haemocytes diameter, cellular proliferation and lysoyme. In all graphs, asterisks indicating statistically significant variations at each sampling time among experimental groups were showed only when significant effects of the factors “treatment” and/or “treatment-time interaction” was revealed by the two-way ANOVA analysis.

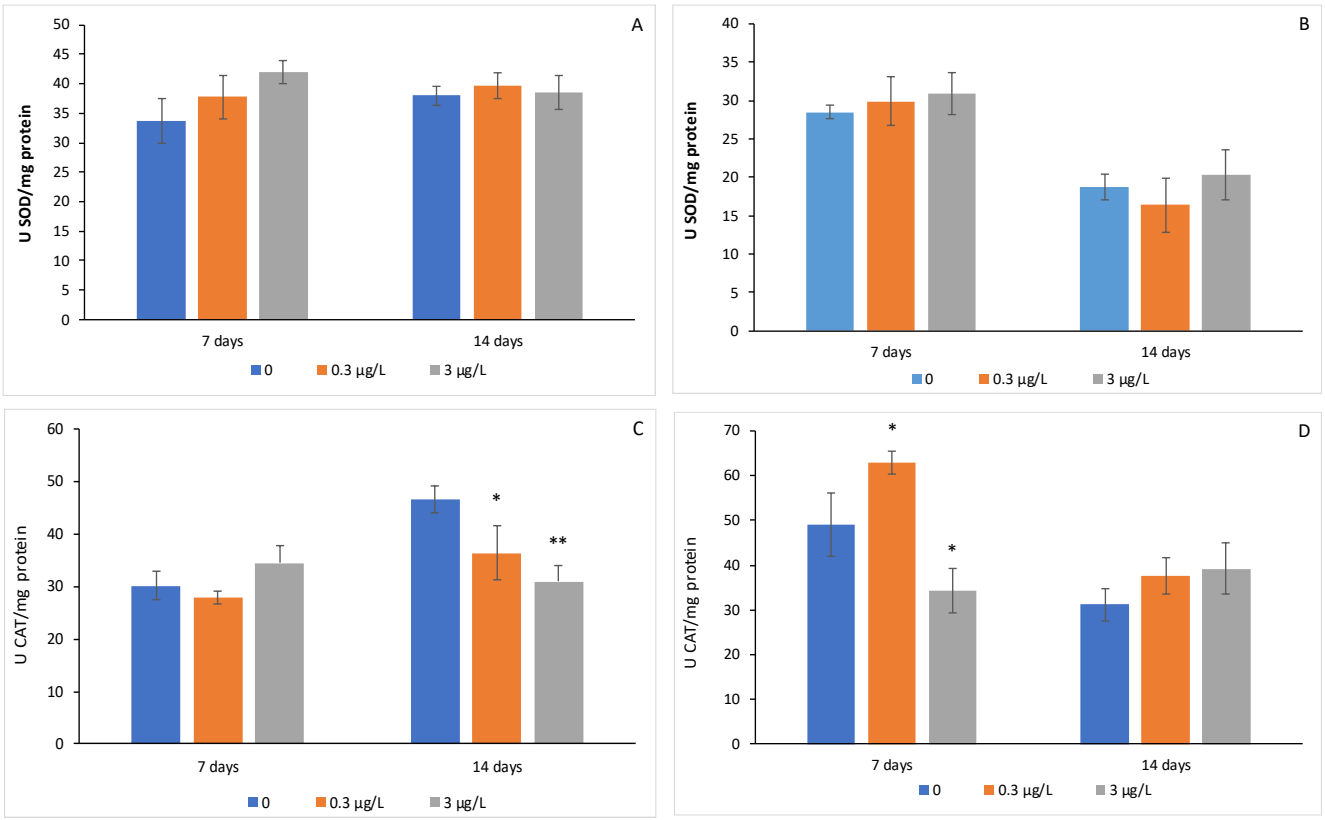

Figure 2. Superoxide dismutase (SOD) and Catalase activities in gill (A, C) and digestive gland (B, D). In all graphs, asterisks indicating statistically significant variations at each sampling time among experimental groups were showed only when significant effects of the factors "treatment" and/or "treatment-time interaction" was revealed by the two-way ANOVA analysis.

Exposure time (F=7.04, p=0.013), treatment (F=13.25, p=0.000) and time-treatment interaction (F=10.15, p=0.001) significantly influenced haemocytes proliferation. The pairwise comparison highlighted a significant (p<0.001) increase in cell proliferation in mussels exposed for 7 days at AS_H (Fig. 1D). Conversely, none of the experimental factors affected LDH activity (data not shown). The factors time (F=9.50, p=0.005), treatment (F=7.21, p=0.003) and time-treatment interaction (F=3.85, p=0.03) influenced significantly HL lysozyme activity, with a significant increase in enzyme activity in mussels exposed for 14 days at AS_H (Fig. 1E).

Exposure to AS did not affect significantly SOD activity in gills, while exposure time influenced enzyme activity in digestive gland (F=25.96, p=0.000) (Fig. 2A, 2B). Gill CAT activity showed a significant decrease in mussels exposed for 14 days to AS_L and AS_H (p<0.05 and p<0.01, respectively). After 7 days of exposure CAT activity in digestive gland increased significantly (p<0.05) in AS_L and decreased significantly (p<0.05) in AS_H when compared to the related controls (Fig. 2C, 2D). None of the experimental factors influenced significantly GST activity of digestive gland (time: F=2.08, p=0.169; treatment: F=1.22, p=0.310; treatment-time interaction: F=1.09, p=0.352) (data not shown), while only exposure time affected significantly both AChE (F=22.17, p=0.000) and BChE in gills (F=39.80, p=0.000) (data not shown).

6.2.7 Microbiota characterization

PCoA analysis performed at species level showed a weak separation along between clams collected at 7 and 14 days along the y-axes (PC2 = 17% variability) (Fig.3A). At both sampling time, a clear separation of mussels exposed to the highest concentration of AS was showed (Fig.3B,3C). Lower microbial diversity (Simpson's Index) and evenness were also observed in mussels exposed to the highest AS concentration at the day 7, while no particular evidences were found at day 14 (Fig.3D, 3E).

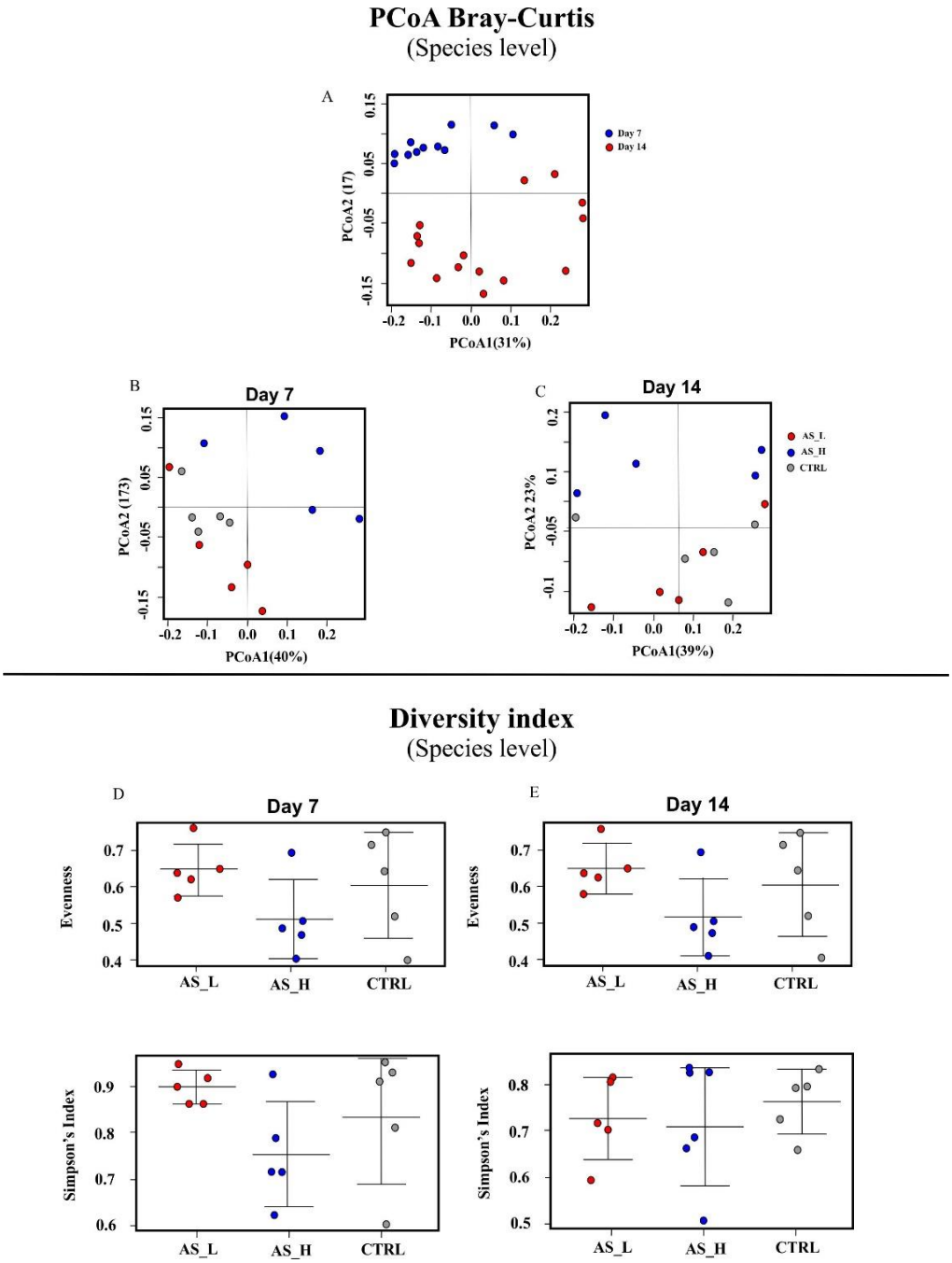

Figure 3. Principal Coordinates Analysis (PCoA) of mussel digestive gland microbiota. A) Different colors indicate the two sampling time (Day 7 and Day 14); B, C) Different colors indicate different treatment considering the whole experiment duration (B), and each sampling time separately (C); D and F) Evenness and Richness diversity index (Simpson's Index) for each group at 7 and 14 days of exposure.

Differences in microbial composition between exposed mussels and CTRL were also explored through one-way ANOVA at species and genus levels (Table 1).

Table 1. Lists of significant genera and species obtained comparing control and exposed mussels AS_L and AS_H after 7 and 14 days of exposure (p-value<0.05). Down- and Up-represented genera/species in exposed mussels are reported in green and red respectively.

AS_L				AS_H			
Genus							
Day 7		Day 14		Day 7		Day 14	
up	down	up	down	up	down	up	down
uncultured_ Grampositive _bacterium	Spongiibacter	Glaciecola	Coxiella		Hyphomonas		
Arcobacter					Spongiibacter		
					Zhongshania		
					Pseudofulvibacter		
Species							
Vibrio_Vibrio _aestuarianus	Spongiibacter_ Spongiibacter_ sp._ IMCC21906	Glaciecola_uncultured_gamma_ proteobacterium _CHABXII8			Hyphomonas_ uncultured_bacterium		
	Neptuniibacter _Raricirrus_ beryli_ associated_ bacterium				Spongiibacter_ Spongiibacter_sp. _IMCC21906		
					Zhongshania_ uncultured_ bacterium		
					Pseudofulvibacter _uncultured_ bacterium		

After 7 days of exposure, 4 microbial species and 3 genera were found altered in mussels treated both with AS_H and AS_L (p-adjusted < 0.05). Among others, mussels treated with AS_L showed over-represented

Vibrio Vibrio aestuarianus species and Arcobacter genus, while AS_H experimental group showed just down-represented taxa compared to the control, like down-representation of *Hyphomonas_uncultured_bacterium*. At day 14, weak microbial changes were observed in mussels exposed to the lowest AS concentration, while no modifications in microbiota composition were observed in mussels digestive gland of AS_H treatment.

6.2.8 Gene expression

PCA revealed clear separation between treatments at 7 and 14 days of exposure (Fig. 4), in particular of AS_H exposed mussels at 14 days. This is 88onfirmed by the highest number of DEGs owned by the long-term AS_H treatment (68 DEGs), followed by AS_L at 7 days of exposures (23 DEGs). It should be observed that within each pairwise comparison, the highest number of DEGs is down-regulated in exposed groups compared to the control.

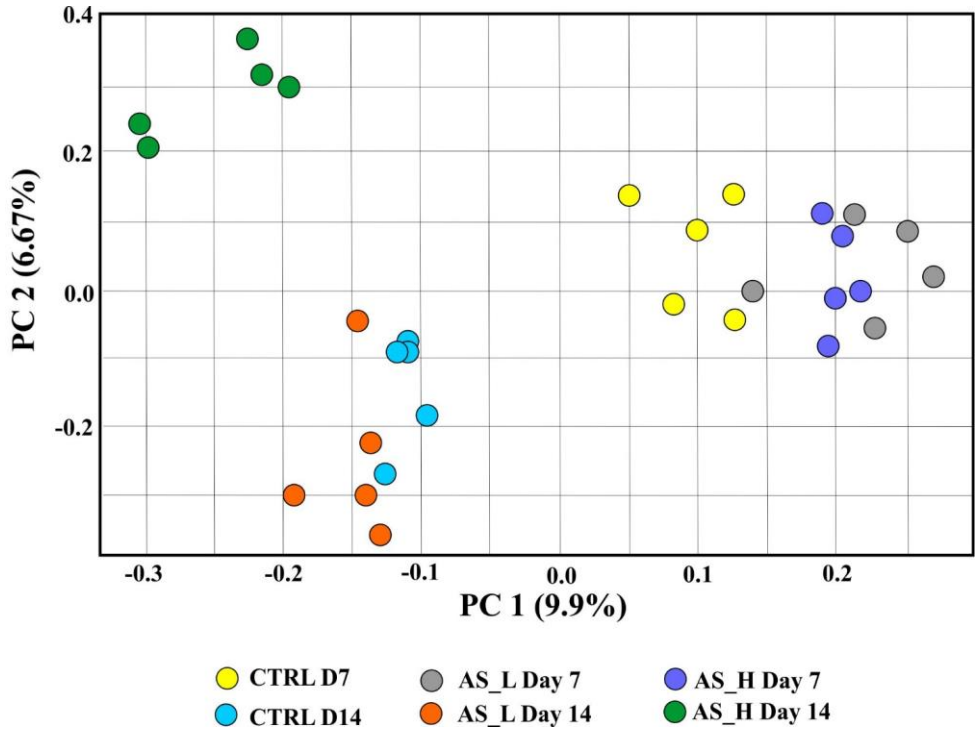

Figure 4. PCA applied to gene expression profiles at each sampling time/concentration of Amyl salicylate.

The majority of genes were uniquely expressed for each treatment/sampling time. Among common DEGs, *Tripartite motif-containing protein 45* (*TRIM45*), a multifunctional protein implicated in transcriptional regulation, was up-regulated in both treatments.

At the day 7, *Heme-binding protein 2* (AS_L) and *Heme-binding protein 1* (AS_H), two genes potentially involved in the promotion of mitochondrial permeability transition and facilitation of necrotic cell death under different types of stress conditions, as well as in the potential removal of toxic compound, were found down-regulated. AS_L treatment showed also several DEGs playing potential role as regulator for calcification of bivalve shells and chitin biosynthesis/modification, such as *N66 matrix protein* (*N66)*, *Gigasin-6*; *Chitin deacetylase 5 isoform A precursor* (*Cda5*). At the same sampling time, mussels exposed to AS_H showed the disruption of *Chitotriosidase-1* (*CHIT1*), a gene likely involved in degradation of chitin-containing pathogens, immune responses and metabolism.

Among DEGs at 14 days, mussels treated with AS_L showed several genes taking part in processes like cells adhesion, cell-collagen, development processes and intracellular signaling, as *Collagen alpha-5(VI) chain* (*COL6A5*) and *Collagen alpha-2(XI) chain* (*COL11A2*). Moreover, up-regulation of *Cyclic AMP-dependent transcription factor* (*ATF-3*), gene induced by a variety of signals with potential role in cellular stress response, and *WASH complex subunit 7*, involved in intracellular transports during stress conditions, were also found. Mussels exposed to AS_H showed alteration of a conspicuous number of genes related to transcriptional regulation, such as *Tripartite motif-containing protein 45* (*TRIM45*), *Histone deacetylase 6* (*HD6*) and *Histone-lysine N-methyltransferase* (*SETD8*). Furthermore, changes in the expression of genes involved in stress and xenobiotic responses, such as genes *Heat Shock Protein 70kD 12B* (*HSPA12B*) and *Glutathione S-transferase* were also found. To conclude, alterations in genes involved in inflammatory/immune/viral responses like *TLR4 interactor with leucine rich repeats* (*TRIL*), *Toll-like receptor 13* (*TLR13*), *Radical S-adenosyl methionine domain-containing protein 2* (*RSAD2*), as well as in regulation of apoptotic processes, like *Poly [ADP-ribose] polymerase 14* (*PARP14*); *Apoptosis inhibitor 5* (*API5*), were also observed. Furthermore, AS_H exhibited a down-regulation of *Solute carrier family 6 member* (*SLC6*) gene playing a role in neuronal amino acid transport.

Results of GSEA analyses are reported in Table 2.The highest number of altered pathways identified through GSEA was found in AS_H treatment. In all treatment, all enriched pathways were found significantly up-regulated, except for “P53 signalling pathway” and “Proteasome” in AS_H treatment at day 14 (Table 2).

Table 2. List of biological processes and KEGG pathways significantly found up-regulated (red) or down-regulated (green) in each treatment/sampling time by GSEA (Adj. p-value<0.1).

	AS_L Day7	AS_H Day7	AS_L Day14	AS_H Day14
ENERGY METABOLISM				
GO:0006754~ATP biosynthetic process				
GO_OXIDATIVE_PHOSPHORYLATION				
KEGG_CITRATE_CYCLE_TCA_CYCLE				
KEGG_fatty acid metabolism				
KEGG_Glycolysis-Gluconeogenesis				
XENOBIOTIC METABOLISM				
HALLMARK_XENOBIOTIC_METABOLISM				
KEGG_ABC_TRANSPORTERS				
KEGG_DRUG_METABOLISM_CYTOCHROME_P450				
STRESS RESPONSE				
KEGG_PATHWAYS_IN_CANCER				
GO_CELLULAR_RESPONSE_TO_EXTERNAL_STIMULUS				
GO_ION_TRANSMEMBRANE_TRANSPORT				
GO_TRANSPORT_VESICLE				
GOLGI_APPARATUS				
KEGG_FOCAL_ADHESION				
KEGG_LYSOSOME				
KEGG_PPAR_SIGNALING_PATHWAY				
KEGG_PROTEASOME				
PROTEOLYSIS				
NEUROTRANSMISSION/SYNAPSE				

	AS_L Day7	AS_H Day7	AS_L Day14	AS_H Day14
GO_GLUTAMATERGIC_SYNAPSE				
APOPTOSIS/CELL DEATH				
GO_AUTOPHAGIC_CELL_DEATH				
GO_REGULATION_OF_CELL_DEATH				
KEGG_APOPTOSIS				
SIGNALING				
KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY				
KEGG_P53_SIGNALING_PATHWAY				
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY				
IMMUNE RESPONSE/INFLAMMATION				
DEFENSE_RESPONSE_TO_VIRUS				
IMMUNE_SYSTEM_PROCESS				
KEGG_ARACHIDONIC_ACID_METABOLISM				

The highest number of enriched pathways related to stress responses were found in all treatments, excepted in AS_L at 7 days. Similarly, several pathways related to xenobiotic metabolism were enriched in all treatments except in AS_L day 7 (like “Drug metabolism cytochrome p450” in AS_L day 14 and AS_H day 14).

In mussels treated with AS_H, several pathways involved in energetic metabolism were enriched and up-regulated. Moreover, AS_H showed an enrichment of “Arachidonic acid metabolism” (day 7), “Defence response to virus” and “Immune system process” (day 14), pathways related to homeostasis and immune response/inflammation. Regarding apoptosis/Cell death, enrichment of “Apoptosis” and “Regulation of cell death” pathways were observed in AS_L (day 7) and AS_H (day 14).

6.3 Discussion

Mussels are filter-feeding sessile animals of high economic interest widely employed in ecotoxicological studies. Mussels has a key role for ecosystem functioning and demonstrated impressive ability to survive in unfavorable conditions as well as to adsorb/bioaccumulate pollutants from the environment (Świacka et al., 2019). The Mediterranean mussel *M. galloprovincialis* is among the most important economic bivalve species in Italy, and it is widely farmed within the Venice lagoon. This area, mainly due to the urban centers of Venice and Chioggia and the industrial area of Porto Marghera, is impacted by several chemicals, potentially affecting the ecosystems and farmed bivalve species. Accordingly, several national and regional monitoring program aiming to detect and quantify emerging contaminants are ongoing. Recent monitoring campaign, performed within the research project Venezia 2021, highlighted the fragrance Amyl Salycilate among the most represented emerging contaminants in Venice lagoon. Studies on the presence, fate, behavior in the environment and ecotoxicity of fragrances have raised only recently. The most of these studies available in literature are focused on musky fragrances such as Galaxolide®, (HHCB) and Tonalide® (AHTN) whose presence in several aqueous systems and aquatic biota is rather well documented (Casatta et al., 2015; Heberer, 2002; Kannan et al., 2005; Rimkus, 1999; Rüdel et al., 2006; Sumner et al., 2010). These chemicals were found in surface and costal waters and in animal tissues in amount of ng L-1 and µg L-1 (review by Prichard and Granek, 2016; Reiner and Kannan, 2011). Few studies investigating the ecotoxicity highlighted chronic, sub-chronic and acute toxicity with lethal and sub-lethal consequences (Dietrich and Hitzfel, 2004;

Ehiguese et al., 2020; Prichard and Granek, 2016) in freshwater and marine organisms, including marine mollusks bivalves (Ehiguese et al., 2020). However, generally, mechanisms of action of fragrances for ecotoxicity is still poorly known (Dietrich and Hitzfel, 2004) and in particular, in this regard, no studies investigated Amyl salicylate, claiming hence further studies urgently.

6.3.1 Presence in water and dose-dependent bioaccumulation capacity of Amyl salicylate

Amyl salicylate is a fragrance whose occurrence and related environmental consequences are not still deeply studied. Nevertheless, this fragrance was found in water of different environments worldwide. To date, monitoring campaign in Venice lagoon highlighted concentrations ranging between 0 and 150 ng/L depending on sampling sites, with Salicylates (Amyl, Hexyl and Benzyl Salicylate) representing the most abundant compounds (Vecchiato et al., 2016). In our study, bioaccumulation in mussels was investigated for the first time considering environmental concentrations detected in Venice lagoon.

Concentrations of AS detected in water of exposed mussels after 24h dropped to levels comparable to the control (3.3-17 ng L-1) suggesting relatively fast kinetics of adsorption/removal of this compound in aqueous solution. Despite AS bioaccumulation was defined for the first time in mussels acclimated for 7 days, its detection in control group at both sampling times confirm the bioaccumulability of this compound in natural environment. Our study also suggested potential dose-dependent bioaccumulable capacity of AS in mussels over the experiment, with AS bioaccumulation at the two concentrations of 0.3 and 3 µg/L increased respectively by about 1.5 and 3 times compared to the control. In addition to this finding, another evidence of the bioaccumulation capacity is notable in mussels of AS_L that exhibited an increase accumulation of the fragrances from 7 up to 14 days of treatment.

Bioconcentration Factor (BCF), higher in mussels exposed to lower concentration, suggested potential dose-dependent AS behaviour. Fragrances are usually lipophilic substances, easily able to be bioaccumulated by marine organism (Liu et al., 2021). Despite the accumulation of few fragrances in tissues of non-target organisms, such as marine bivalves, are quite well documented (Aminot et al., 2021), studies on the uptake of Amyl salicylate in shellfish and other marine organisms remains still unknown.

6.3.2 Time-dependent responses: Short-term exposure to AS and biochemical changes in mussels

Among the main results of this study, biochemical investigations highlighted underlying time-dependent responses of mussels. The study of biomarkers revealed significant alterations in THC, diameter, volume and proliferation of haemocytes in mussels treated for 7 days with the highest AC concentration. Several studies showed the immunotoxicity on haemocytes of bivalves following emerging contaminants exposure and alterations in THC and haemocytes proliferation were already observed in ecotoxicological studies on bivalves treated with PCPPs for 7 days. Among them, decreased THC was observed in mussels treated with a mixture of CECs, that herbicide, synthetic estrogen and Amyl salicylate (Fabrello et al., 2021). Moreover, alterations in haemocytes were observed also in clams exposed to Triclosan (Matozzo et al., 2012a), an antibacterial agent reported in the PPCPs list. Overall, the alteration of the number, diameter, volume and proliferation of haemocytes suggests potential ability of AS to trigger disorders in immune system of mussels being haemocytes the first cells prone to act the first protection against different stressors (Mayrand et al., 2005; Zannella et al., 2017). Haemocytes are susceptible targets of many contaminants, with serious consequences for the homeostatic capacity of mollusks with following potential disruptions in the correct functioning of the immune system (Auffret and Oubella, 1997; Zannella et al., 2017). In turn, physiological alterations of mussels, especially in immune system, may lead to the loss of the ability to face modification

in microbiota community and limiting barriers to the spread of opportunistic pathogens (Burgos-Aceves and Faggio, 2017; Zannella et al., 2017).

6.3.3 Time- and dose-dependent responses: Short-term and low-concentration exposure to AS affected the microbiota community of mussels

Microbiota community in marine benthic filter-feeder organisms plays a crucial role because it is involved in nutrient acquisition, processing of metabolic waste by eliminating it via the biotransformation of several toxic metabolites and production of special secondary metabolites (Evariste et al., 2019; Santisi et al., 2015).

Despite the exposure to AS induced not extremely severe repercussions in gut microbial composition of mussels, the first worthy finding regards the time-dependent responses of the gut microbiota of mussels showing the main effects in the early stage exposure (7 day). In this regard, one of the most interesting results is the down-representation of *Hyphomonas_uncultured_bacterium* species in AS_H treatment. In general, this taxon is known to be able to deaminate amino acids or to oxidize the tricarboxylic acid cycle intermediates as the major catabolic pathway for energy and growth (Whitman et al., 2015), even if not studied in relation with Amyl salicylate exposure yet. However, the association *Hyphomonas_uncultured_bacterium-M. galloprovincialis* as well as the relation between the bacterium with environmental pollution have not been investigated so far, so the development of futher studies might shed more light on these topics.

No taxa were found significantly changed in AS_H after 14 days and a lower number of bacteria in AS_L at the day 14 was observed compared to the short-term treatment. The greatest number of alterations in short-term exposure might suggest a peculiar time-dependent response. Because of their immobilization, in fact, metabolic responses of sessile marine mollusks depend on the duration of the stimulation (Livingstone, 1981; Ramos-Martínez et al., 1993).

A similar result of short-term effects was observed in Bernardini et al. (2021) where clams treated with Perfluoroalkyl chemicals exhibited none alterations in microbiota community in the majority of treatments in the longest-term exposure. Furthermore, higher microbial diversity was found in gut samples of *M. galloprovincialis* within short-term exposure to warm temperature, an environmental stress condition, compared to the long-lasting treatment (Li et al., 2019).

Furthermore, noteworthy, an important finding is represented by the spread of opportunistic pathogens in the treatment to the lowest concentration of AS.

After 7 days of exposure to AS_L, mussels exhibit the over-representation of the potential opportunistic pathogens, Arcobacter genus, and the shellfish pathogen *Vibrio_Vibrio_aestuarianus* species. These Gram-negative bacteria are widely present in marine environment that have been already shown to be present in moribund and unhealthy bivalves exposed to stressful environmental conditions, included environmental pollution (Bernardini et al., 2021; Iori et al., 2020; Milan et al., 2018). Vibrio ad Arcobacter are potentially able to cause serious diseases in bivalves organisms leading them up to the death (Beaz-Hidalgo et al., 2010; Vezzulli et al., 2010). In particular, *Vibrio aestuarianus*, well-known to be pathogenic for shellfish (Tison and Seidler, 1983), was shown associated to the mortality events of oysters (Saulnier et al., 2009) as well as able to impair the functional response of the haemocytes and secrete extracellular products including a metalloprotease in Pacific oyster (*Crassostrea gigas*) (Labreuche et al., 2006,2010). Moreover, in *Mytilus galloprovincialis* it was observed to cause immunotoxicity (Balbi et al., 2013) and infections, even if moderate, as well as mortalities in animals with a high bacterial concentration in adverse environmental conditions (Romero et al., 2014).

Overall, the study of host-microbiota interactions, however, represents one of the most complex and reliable topic for understanding the role of bacterial communities in the host where they may lead to potential biochemical and molecular changes (Santisi et al., 2015). To our knowledge, still few studies investigated the role of environmental emerging pollutants, like PPCPs, in the affection of the microbiota in marine mollusks and their consequences to the host-heath. The improvement of molecular techniques especially high-throughput next generation sequencing (NGS) technologies targeting the bacterial 16S rRNA gene, occurred in the last years, has eased the study of host-microbiota interaction in mollusks where the type of mutualistic/pathogenic interactions may vary depending on the surrounding environment (Destoumieux-Garzón et al., 2020; Pierce and Ward, 2018). Thus, the application of a multidisciplinary approach allowed providing the first evidences of potential consequences of AS fragrance in an ecological key species by now proven as model species in ecotoxicological studies. Nevertheless, to our knowledge, none study investigated the interaction host-microbiota following the exposure to fragrances, so no deeply comparisons can be done with our study. This lack of information prompts and encourage the scientific community to perform more researches in this regard.

6.3.4 Transcriptional responses to both concentrations of AS

The ability of organisms to keep their heath safe in hostile conditions is defined by the capacity to quickly address the changing environment. In the present study, considered the effects in gut microbiota alterations and transcriptional modifications in the host, the time- and dose- dependent responses discussed in the previous paragraphs might be partially explained by the putative ability of mussels to recover and to re-establish the gut flora and potentially limiting biochemical changes after a long exposure period. Molecular modulations may represent the triggering point in response to the presence of pollutants and stressful conditions that precedes the effective toxicity (Adams et al., 1989; Bernardini et al., 2021).

A quite common result to all experimental groups was the down-regulation of the majority of DEGs. A possible explanation of this result is a process called Stress-Induced Transcriptional Attenuation (SITA), consisting in molecular responses to protect cells from damage through a rapid genes down-regulation (Aprile-Garcia et al., 2019). Despite the overall process is still not clear, this molecular regulation has been explained as a possible increased energy allocation for the protection against the environmental stress instead for from growth-promoting anabolic activities through the down-regulation of genes involved in different cellular processes (Aprile-Garcia et al., 2019; Gasch et al., 2000; Mahat et al., 2016).

At the 7th day of exposure, genes involved in Heme binding proteins composition were found altered in both experimental groups. Heme binding proteins is a group of proteins that act as facilitators of intracellular transport of cytosolic proteins due to their high affinity for the heme group (Iwahara et al., 1995). Among the heme-composed proteins, Cytochrome (CYP) P450s superfamily, ubiquitously distributed in all biological organisms, deserves to be cited. CYP450s is a large group of heme-containing enzymes that catalyze a wide range of chemical reactions (Li et al., 2008). It has an important role in the major phase I-type classes of detoxification enzymes found in terrestrial and aquatic organisms ranging from bacteria to vertebrates (Zanette et al., 2010). Despite it is still poorly known in bivalves, few enzymes belonged to the CYP450 superfamily were shown to be involved in a variety of detoxification processes and in response to xenobiotic exposures in marine invertebrates (Snyder, 2000; Zanette et al., 2010), including mussels exposed to musky fragrances (Focardi et al., 2011). This evidence might find a link to the up-regulation of the KEGG pathway "Drug metabolism cytochrome P450" in AS_H day 7, enriched also in mussels exposed to the low dose of AS for 14 days. Furthermore, among DEGs related to responses to stress condition, *WASH complex subunit 7* was found altered in this experimental group. This is a gene belonged to the WASH complex, which functions in the intracellular transport of endosomes that suppose a potential action during stressful conditions.

Moreover, genes like *HSPA12B*, encoding for general responses to stress, and *ATF-3*, that may have a pivotal role in many viral and cellular promoters (Hai et al., 1999), were found altered. While the role of Heat Shock Proteins in stress response of bivalves was testified by several studies (e.g. Cruz-Rodríguez and Chu, 2002; Izagirre et al., 2014; Mezzelani et al., 2021; Song et al., 2006), the modulation of *AFT-3* in marine mollusks following the exposure to contaminants was observed just in gills of mussels (*Mytilus californianus*) treated with low doses of copper to our knowledge (Hall et al., 2020). *ATF-3* can act as either repressors or activators of transcription. *ATF3* expression is maintained at low levels in quiescent cells, whose modulation may be induced by a wide variety of signals including those initiated by cytokines, genotoxic agents, or physiological stresses (Hall et al., 2020; Thompson et al., 2009).

Similar to AS_L treatment, mussels of AS_H groups showed several enriched pathways involved in xenobiotic metabolism and stress responses both at 7 and at 14 days of exposure, suggesting potential direct consequences of high concentrations of AS regardless the time of exposure. KEGG pathway "PPAR signalling pathway" and "Proteasome" were found altered in both experimental groups compared to the control. Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that are activated by fatty acids and their derivatives. In mammals, PPARs act regulating the expression of several lipid regulatory proteins modulate the expression of genes involved in lipid metabolism, maintenance of metabolic homeostasis and inflammation (Canesi et al., 2007; Staels et al., 1998). Results obtained by the study of Cajaraville and Ortiz-Zarragoitia (2006) confirm the use of peroxisome proliferation, modulated by the PPARs, as a specific biomarker of exposure to contaminants such as hydrocarbons, phthalate plasticizers and polybrominated flame retardants in adult blue mussels (*Mytilus edulis*), in accordance to several studies published over the years. Furthermore, the enrichment of these pathways, together with the overregulation of "Cellular response to external stimulus", "Xenobiotic metabolisms" and other enriched pathways, may be corroborated by the altered expression of few genes like *HSP70* and *GST* involved in general responses and detoxification processes detected in this treatment.

Thus, all this considered, our results suggest a wide ability of both concentrations of AS to modulate genes often involved in stress response. Nevertheless, specific responses to the different concentrations of AS were also found and discussed below.

6.3.5 Dose-dependent response

An interesting specific result obtained after 7 days regards mussels of AS_L treatment, where several genes playing a role in processes of calcification in the shells of mollusks and chitin biosynthesis and/or modification were found altered. Bivalves have external mineral shells, deputed to protect soft tissue of mollusc from predation and desiccation that may be affected by environmental changes, including exposure to hazardous chemicals (Harayashiki et al., 2020). In this regard, over the decades, several studies testified the effects of contaminants on shell calcification anomalies. Alzieu (2000, 1998) showed general outbreak of shell calcification anomalies in adult oysters from the Arcachon Bay in France due to the contact with Tributylin, an anti-fouling paints. Miglioli et al. (2021) showed that Bisphenol A, an emerging contaminant of high concern, induced concentration-dependent alterations in shell-calcified shell at different larval stages of specimens of M. galloprovincialis. Furthermore, at 48 hours post fertilization, a selective down-regulation of genes involved in shell formation was observed in embryos of M. galloprovincialis exposed to Bisphenol A and polystyrene nanoparticles (Balbi et al., 2017, 2016). As in studies of Balbi and colleagues, in the present research genes involved in shell calcification/development were found down-regulated indicating potential alterations in $CaCO_3$ deposition and mineralization in mussels following the exposure to Amyl salicylate.

Furthermore, the alteration of genes at 14 days in AS_L involved COL6A5 and COL11A2. Collagen is the most abundant high-molecular-weight extracellular protein with specific amino acid composition in both

invertebrate and vertebrate organisms comprising about 25–30% of total proteins (Rodríguez et al., 2017). Furthermore, it has the role of adhesive plaques of the byssus representing, together with elastic fibers, the main structural element of this product (Gosling, 2007). The byssus has several key roles during the different life stages of mussels, like in the settlement, metamorphosis, morphological evolution, adhesion to hard substrates and adaptive radiations of epifaunal species (McCartney, 2021). Thus, alterations of the collagen production might induce an important impairment in mussels with following consequences in the correct functioning of several aspects of mussels' life.

Overall, despite still little studies are available, the reported results of the present research revealed a potential complexity in shell/byssus formation under stress conditions due to the presence of AS fragrance, that, influencing the stability of cytoskeleton and cell structures in mussels, suggests potential developmental effects of CECs (Khan et al., 2020; Yu et al., 2016).

Concerning exposures to the highest AS concentration, few differently expressed genes participate in responses to stress and in defence against pathogens at 7 days AS_H, such as CHIT1 (AS_H).

CHIT1 is an enzyme that breaks down chitin (Feis et al., 2018) and in mammals it acts in innate immune responses, playing a pivotal role in infectious disease, like malaria and fungi infections as a host defence towards chitin in pathogen's cell structure (Kanneganti, 2012), suggesting an active role in maintaining the homeostasis in immune system (Elias et al., 2005). The presence and the involvement of CHIT1 in immune responses and in inflammatory processes was previously observed in mammalian, fishes, and invertebrates (reviewed in Watanabe et al., 2018), like in the common octopus (Ogino et al., 2014), shrimps (Niu et al., 2018) and blackfoot pāua (Neave et al., 2019). A modified CHIT1 regulation was detected blue mussels (Mytilus edulis) after intestinal parasite infections, suggesting a role in direct coupling of gene expression between hosts and parasites and the regulation of inflammatory processes in marine bivalves (Feis et al., 2018). Potential correlated alterations in immune responses and inflammation in AS_H day 7 might also corroborated by the previously discussed results of biomarkers and in the up-regulation of KEGG pathway "Arachidonic acid metabolism". This pathway describes the production and subsequent metabolism of arachidonic acid, an omega-6 fatty acid, present in the phospholipids of membranes of the body's cells. Several products of this pathways act modulating the activities of ion channels, protein kinases, ion pumps, and neurotransmitter uptake systems, affecting processes such as cellular proliferation, inflammation, and homeostasis (National Center for Biotechnology Information, 2021).

At day 14, AS_H group showed alterations in genes potentially involved in apoptotic processes, such as PARP14 and API5. API5 is a gene encoding an apoptosis inhibitory protein that suppresses the transcription factor E2F1-induced apoptosis and also interacts with a nuclear factor involved in apoptotic DNA fragmentation. Modulation of API1 was reported to be altered following the infection by Avibirnavirus and to induce dendritic cell and immune response activation via Toll like receptor 4 (TLR4) (Deng et al., 2021; Kim et al., 2018; Mayank et al., 2015). In the present study, mussels of AS_H treated for 14 days, in fact, showed a simultaneous alteration of API5 and TLR4, confirming the potential link between the two genes. This result may find a further proof in the results from GSEA that showed an enrichment of GO pathways involved in apoptosis/cell death and, again, in immune response/inflammation. Moreover, it seems to find a potential link even with the altered regulation of TLR13, in the enriched pathway "Signalling", likewise found uniquely altered in AS high dose treatment.

Another interesting result is in the alteration of several genes involved in signal transduction and transcriptional responses. Among these, TRIM45 was found up modulated. TRIM proteins family has been implicated in many biological processes including cell differentiation, apoptosis, transcriptional regulation and signaling pathways (Zhao et al., 2014). More in deep, TRIM45 is a member of the tripartite motif family that encodes protein that may function as a transcriptional repressor of the mitogen-activated protein kinase (MAPK) pathway, an important mediator of signal transduction activated by a variety of stimuli, such as

environmental stresses (Wang et al., 2004). Transcriptional analyses performed in the Pacific oyster (Crassostrea gigas) inoculated of Ostreid herpesvirus 1 mVar (OsHV-1mVar) revealed modifications in several genes belonging to the TRIM family as response for the inhibition of viral transcription (He et al., 2015) suggesting a potential role also in innate immune response during stress conditions (Jouaux et al., 2013). Furthermore, few genes of TRIM family, included TRIM45, were defined included in the list of genes deputed in the adaptation to hypo-osmotic conditions in oysters (Zhao et al., 2014).

An up-regulation of other two genes involved in transcriptional responses was found in HD6 and SETD8. HD6 is a protein-coding gene that plays a pivotal role the deacetylation of lysine residues on the N-terminal part of the core histones, participating in transcriptional regulation, epigenetic repression, cell cycle progression and developmental. Similarly, SETD8 represents a specific tag for epigenetic transcriptional repression and plays a central role in the proliferation of cells, probably by contributing to the maintenance of proper higher-order structure of DNA during mitosis, DNA replication and damage and cell cycle regulation, both in physiological and pathological conditions (Milite et al., 2016). These findings accord with data from literature where alteration of transcriptional co-activators/co-repressors related to the marine environment has been widely demonstrate.

Finally, a specific modification of "Glutamatergic synapse" enriched pathway involved in neurotransmission/synapse emerged in the long-lasting treatment with AS_H. This may find a potential link to the alteration of SLC6 gene. In mammals, SLC6 is a gene that play critical roles in neurotransmission, cellular and whole body homeostasis (Bröer and Gether, 2012; Pramod et al., 2013) and control transmembrane movement of many types of substrates, including drugs and toxins (Hermann et al., 2020). Even if still poorly studied so far, the expression of genes related to the SLC family were observed in marine organisms, like in the scyphozoan jellyfish Cyanea capillata (Bouchard et al., 2019), during a stress condition, like in embryos of Olive flounder (Paralichthys olivaceus) treated with Irgarol 1051®, an alternative antifouling biocides (Moon et al., 2019). However, in contrast to the hypothesis of neurotoxicity of fragrances, Rilievo et al. (2021) did not evidence any particular neurotoxic effects of HHCB in Manila clam (Ruditapes philippinarum). Nevertheless, neurotoxicity of PPCPs and fragrances has not been well studied yet, mostly in shellfish organisms and a deepening is required in order to not to exclude the potential neuro-consequences of fragrances on marine organisms.

6.4 Conclusion

Amyl salicylate is a fragrance largely produced and used on a worldwide scale which environmental fate overlaps that of other Pharmaceuticals and Personal Care Products. Despite its presence was detected in water of several different marine environments including the Venice Lagoon, there is still a lack of knowledge on its potential ecotoxicological risk arousing hence emerging concern. Thus, to our knowledge, the present work represents the first investigation that provided information on a multitude of disciplines linked each other. Our results highlighted responses to the exposure to AS in marine bivalves depending on the time and the dose of exposure. Following the proven presence in the waters of the Venice Lagoon, AS was shown to be bioaccumulated by mussels depending on the level of exposure with following consequences at cellular, biochemical and molecular level. Time-dependent responses were detected at both level of biological complexity. Firstly, the shortest-lasted treatment induced immunotoxicity in haemocytes, the first barrier against stressors. Secondly, considered that results of microbiota community showed the greatest number of species/genera altered in mussels following the short-term treatments, including the potential spread of opportunistic species, it might suggest early responses to stressful conditions due to the alteration of gut microbial taxa. Furthermore, at transcriptional level, specific and common molecular responses based on the concentration of AS highlighted transcriptional alterations of several genes. The majority number of altered

genes were detected in mussels exposed to the highest concentration of AS showing also specific changes in regulation of genes related to immune and transduction/transcriptional responses, apoptosis and neurotoxicity. Conversely, low dose induced modifications in the expression of genes related to shell calcification, chitin and collagen regulation. Common responses, however, were also etected at molecular level, such as xenobiotic and stress responses and the down-regulation of the majority of DEGs.

All these considerations suggest potential ability of AS to stress treated animals, inducing general and specific responses based on the time and the dose of contact to this chemical. However, this first assessment represent only preliminary results on the ecotoxicity of AS thus further studies on edible

marine filter-feeder bivalves need to be carried out in order to shed more light on the real risk of this compound on non-target edible species.

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7. Conclusions

Studies on the presence and ecotoxicity performed in Venezia2021 context provide new information regarding the contamination of waters and first evidences on the effects of these emerging contaminants in Manila clams (*R. philippinarum*) and Mediterranean mussels (*M. galloprovincialis*), edible filter-feeder bivalve mollusks with relevant ecological and commercial role in the Lagoon. The application of a multidisciplinary approach characterized by chemical, biochemical, cellular and molecular (RNA-seq and 16S) analyses allowed us to investigate the health status of treated bivalves and host -microbiota interactions following the exposure to aforementioned compounds. Overall, our results suggest that the physiological status may be compromised following the exposures to these emerging contaminants through their direct effects on key molecular pathways or following the spread of opportunistic pathgens. These studies provide important information on the occurrence of emerging contaminants and related effects on organisms in the Lagoon of Venice, highlighting the urgent need to extend these investigation to others chemicals detected in Venice lagoon to determine their environmental hazard and risks for human health.