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The effects of glyphosate and AMPA on the mediterranean mussel *Mytilus galloprovincialis* and its microbiota

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ABSTRACT

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.

1. 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, 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 and Powles, 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

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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., 2018).

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.

2. Material and methods

2.1. Bivalve collection and experimental design

This experiment was performed in October 2017 at the Department of Biology, University of Padua (Italy). 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 (Supplementary File S1), and digestive glands were excised and frozen in liquid nitrogen and stored at −20 °C for gene expression and microbiota analyses.

2.2. RNA extraction and library preparation

Total RNA was extracted from 6 individuals for each condition (see Supplementary File S1) 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, 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 'Version 2' 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., 2018. 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 2 × 300. 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). Total number of reads obtained for each sample for microbiota analyses and RNA-seq are reported in Supplementary File S1.

2.3. 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 and Durbin, 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. Geneexpression data were then analysed with Multi Experiment Viewcluster 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).

2.4. Microbiome 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 and Standley, 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.

2.5. qPCR for total bacteria and Vibrio quantification

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 analysed 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- GGACT ACCAGGGTATCTAATCCTGTT, 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 CCGA TAACGAACGAGACTC and bivalve_uni_Rev CACAGACCTGTTATTG CTC.

3. Results

3.1. 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 reported in Supplementary File S2 and summarized in Table 2 and Supplementary File S3. Venn diagrams were then constructed to represent the significant transcripts that are differentially regulated in common across different treatments and time points (see Fig. 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 (Fig. 1 and Supplementary File S3).

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, Supplementary File S3). 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

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 identified for each treatment are reported in Supplementary File S2 and S3.

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*
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*

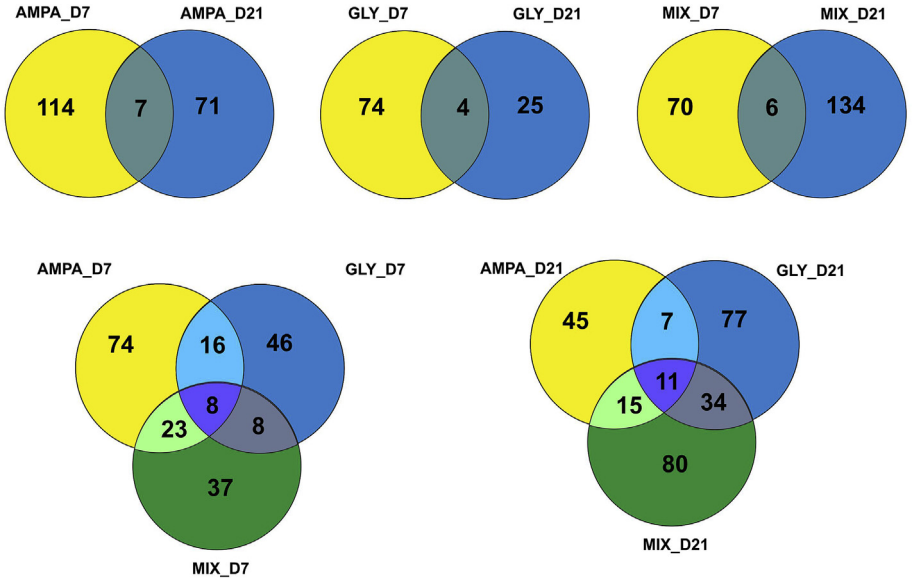


Fig. 1. Venn diagrams reporting the number of differentially expressed genes for each pairwise comparison among control and exposed groups.

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.

3.2. 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%). The overall distribution of classes for each sample group is reported in Supplementary File S4. 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; Fig. 2A).

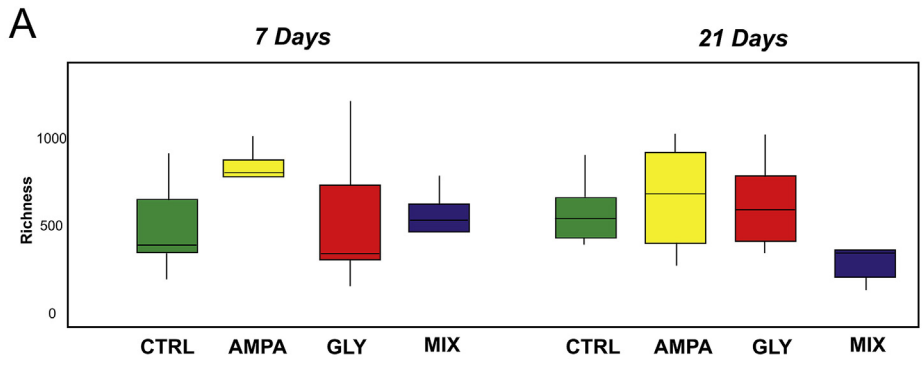
Mussels exposed to GLY significantly differed in the abundance of 17 and 19 ASVs after 7 and 21 days respectively, compared to CTRL. Among them, 8 and 10 ASVs were present in greater abundance in GLY after 7 and 21 days, compared to CTRL (Supplementary File S5; log2 FC 5.8 to 23.2), including five members from the genus *Vibrio* at 7 days. After 21 days, in mussels exposed to GLY ten ASVs were significantly more abundant (Supplementary File S5; 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).

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.

3.3. qPCR for total bacteria and Vibrio quantification

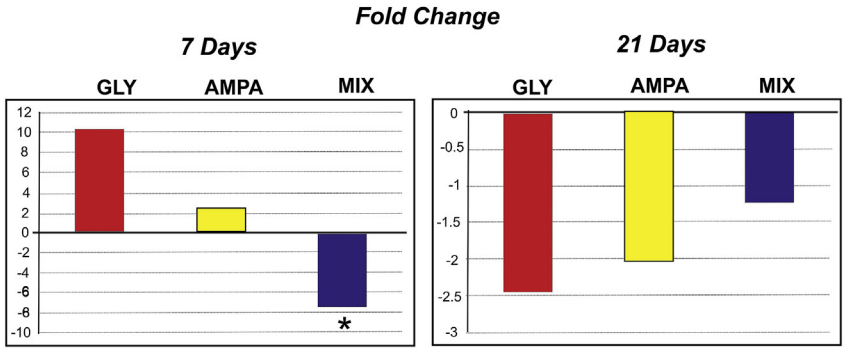
To determine the effects of GLY, AMPA and MIX on the size of the gut microbiome, relative abundances of digestive gland bacteria were assessed through quantitative PCR (qPCR) applied to total 16S rRNA. Considering that the *Vibrio* genus include species pathogenic to bivalves that are known to be associated with abnormal mortality outbreaks in farmed animals (e.g. King et al., 2012; Petton et al., 2015; Le Roux et al., 2016), total *Vibrio* load was also investigated. Likely due to the high inter-individual variability (Fig. 2B and C), 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-value < 0.05; Mann-Whitney test; Fig. 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



B

Bacterial 16S relative quantification ($2^{-\Delta\Delta CT}$)

	CTRL	GLY	AMPA	MIX
7 davs	27.86±14.21	285.93±550.75	67.99±76.64	3.76±2.17
21 davs	101.32±145.42	41.51±59.17	50.13±110.02	82.36±166.5



C

Total Vibrio 16S relative quantification ($2^{-\Delta\Delta CT}$)

	CTRL	GLY	AMPA	MIX
7 davs	17.18±18.23	32.36±33.93	40.00±74.49	24.48±48.98
21 davs	304.24±349.82	31.26±43.9	273.07±630.3	761.08±1424.19

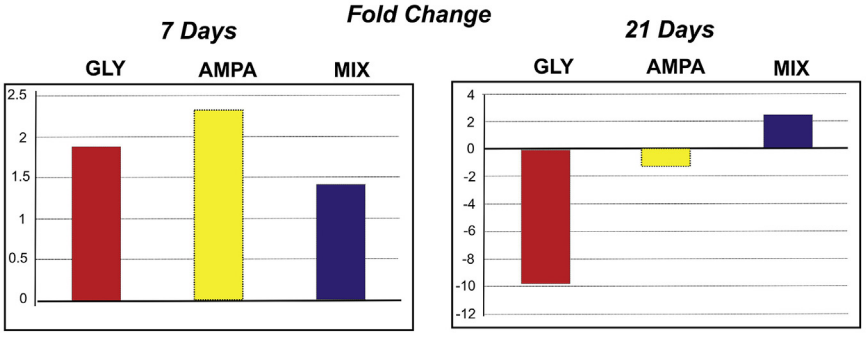


Fig. 2. (A) Richness diversity index for each group at 7 and 21 days. CTRL: control group; GLY: glyphosate exposure; MIX: AMPA + GLY; **(B and C)** Relative quantification of total bacteria (B) and total *Vibrio* load (C) obtained by qPCR. qPCR was performed in the same individuals considered for gene expression and microbiota analyses. The 2-ΔΔCq reported in Tables 2B and 2C represent the mean value and the standard deviation of the 6 samples analysed 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).

decrease of total *Vibrio* load in GLY-exposed mussels at 21 days compared to the CTRL group (Fig. 2C).

4. 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.

4.1. 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., 2018).

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 (Fig. 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.

4.2. 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 and LaBanca 2003; Donaghy et al., 2009; Sokolova, 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., 2018). 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 and Song, 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).

4.3. 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, 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, 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 signalling of glycine, was also observed in response to GLY in our previous studies (Milan et al., 2018, 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 signalling 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, 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 (Supplementary File S3), plays a pivotal role in detoxification of xenobiotic compounds including organophosphorus compounds (Fujioka and Casida, 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, 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, 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.

5. 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.

Author information

The authors declare no competing financial interest.

Author contributions

MM, VM, BC, TP, and LB conceived and designed the project. MM, VM, LM, JF, MGM, and MS participated in sampling activities and lab controlled exposures. SI, GDR performed RNA extractions and libraries preparation. MM, SF, EG and SI executed all gene expression statistical analyses. MM, SI and EG performed functional annotation analyses. LE, LC, BC and MB performed microbiota analyses. MM, LE, SI, GDR and MS wrote the manuscript. All listed authors edited the manuscript. All authors read and approved the manuscript.

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Data accessibility

All sequencing files are available in NCBI Sequence Read Archive: BioProject PRJNA561723 (RNaseq data); BioProject PRJNA562940 (microbiota sequencing).

Declaration of competing interests

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

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Appendix A. Supplementary data

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