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Environmental Toxicology and Pharmacology

Glyphosate and its breakdown product AMPA elicit cytoprotective responses in haemocytes of the Mediterranean mussel (*Mytilus galloprovincialis*)

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Keywords:	Haemocyte; ABC transporters; Marine mussels; Glyphosate; AMPA; Multixenobiotic resistance system
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First Author:	Rajapaksha Haddokara Gedara Rasika Wathsala
Order of Authors:	Rajapaksha Haddokara Gedara Rasika Wathsala Elena Catasús Folgueras Letizia Iuffrida Marco Candela Roberto Gotti Jessica Fiori Silvia Franzellitti, PhD
Abstract:	<p>This study investigates the effects of glyphosate (GLY) and its metabolite AMPA on cytoprotective and detoxification mechanisms in haemocytes of <i>Mytilus galloprovincialis</i>. Cells were treated <i>in vitro</i> with 0.1 and 1.0 µg/L GLY, 0.1 µg/L, 0.1 and 1.0 µg/L AMPA, or two mixtures GLY+AMPA (0.1 µg/L GLY + 0.1 µg/L AMPA, 1.0 µg/L GLY + 1.0 µg/L AMPA). GLY and AMPA increased MXR efflux activity and modulated expression of the ABCB transcript encoding a MXR related ABC transporter P-glycoprotein. The mixtures GLY+AMPA reduced efflux activity with ABCB down-regulation (at 1 µg/L GLY/AMPA). Modulation of lysosomal and immune related transcripts generally agree with known effects of the chemicals on these physiological functions. Given their cumulative action as chemosensitizers of the MXR system, and their interactive effects on haemocyte parameters, glyphosate and AMPA at environmental concentrations should be addressed as a concern factor for the biological vulnerability of marine habitats.</p>
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DEPARTMENT OF BIOLOGICAL, GEOLOGICAL,
AND ENVIRONMENTAL SCIENCES

Ravenna, 12th June 2022

To Michael D. Coleman, Editor of Environmental Toxicology and Pharmacology

Dear Dr Coleman,

We are pleased to submit our manuscript entitled, **Glyphosate and its breakdown product AMPA elicit cytoprotective responses in haemocytes of the Mediterranean mussel (*Mytilus galloprovincialis*)**, authored by Rajapaksha Haddockara Gedara Rasika Wathsala, Elena Catasús Folgueras, Letizia Iuffrida, Marco Candela, Roberto Gotti, Jessica Fiori, Silvia Franzellitti, as a Research paper for Environmental Toxicology and Pharmacology.

The paper investigates the *in vitro* effects of glyphosate, AMPA, and their mixture (the most environmentally realistic exposure scenario) on cytoprotective/detoxification mechanisms expressed in haemocytes of marine mussels. Since recent studies indicate that ABC transporters may represent a main mechanism of resistance to glyphosate exposure in mussels, our *in vitro* assay was set to assess transcriptional changes of the ABCB gene encoding the mussel ABC transporter P-glycoprotein, and global Multixenobiotic Resistance (MXR) efflux activity, along with transcriptional profiles of lysosomal and immune related gene products. Overall, results show that haemocyte treatment with AMPA and a mixture GLY+AMPA have a limited global impact compared to GLY treatment. As for AMPA this finding agrees with a general assumption that the metabolite is less toxic than the parental compound, in the mixture GLY+AMPA cumulative or interactive are hypothesized.

This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. All the authors have approved the manuscript and agree with submission to your journal. There are no conflicts of interest to declare.

We do hope the manuscript meets the requirements for publication on Environmental Toxicology and Pharmacology.

Looking forward to receiving your comments,

Best Regards

Dr Silvia Franzellitti, PhD



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Highlights

- *Mytilus galloprovincialis* haemocytes were treated in vitro with glyphosate (GLY), AMPA, and GLY+AMPA.
- GLY and AMPA increased MXR efflux activity and modulated *ABCB* expression
- The mixtures GLY+AMPA reduced efflux activity with *ABCB* down-regulation (at 1 µg/L GLY/AMPA).
- Modulation of lysosomal and immune related transcripts generally agree with known effects of the chemicals.
- Acting as chemosensitizers of the MXR system, GLY and AMPA may rise biological vulnerability of marine habitats.

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**Glyphosate and its breakdown product AMPA elicit cytoprotective responses in
haemocytes of the Mediterranean mussel (*Mytilus galloprovincialis*)**

Rajapaksha Haddokara Gedara Rasika Wathsala^{1,2}, Elena Catasús Folgueras¹, Letizia Iuffrida^{1,5}, Marco Candela^{3,5}, Roberto Gotti⁴, Jessica Fiori⁴, Silvia Franzellitti^{1,5,*}

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Abstract

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3 This study investigates the effects of glyphosate (GLY) and its metabolite AMPA on
4 cytoprotective and detoxification mechanisms in haemocytes of *Mytilus galloprovincialis*.
5 Cells were treated *in vitro* with 0.1 and 1.0 µg/L GLY, 0.1 µg/L, 0.1 and 1.0 µg/L AMPA, or
6 two mixtures GLY+AMPA (0.1 µg/L GLY + 0.1 µg/L AMPA, 1.0 µg/L GLY + 1.0 µg/L
7 AMPA). GLY and AMPA increased MXR efflux activity and modulated expression of the
8 *ABCB* transcript encoding a MXR related ABC transporter P-glycoprotein. The mixtures
9 GLY+AMPA reduced efflux activity with *ABCB* down-regulation (at 1 µg/L GLY/AMPA).
10 Modulation of lysosomal and immune related transcripts generally agree with known
11 effects of the chemicals on these physiological functions. Given their cumulative action as
12 chemosensitizers of the MXR system, and their interactive effects on haemocyte
13 parameters, glyphosate and AMPA at environmental concentrations should be addressed
14 as a concern factor for the biological vulnerability of marine habitats.
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43 **Keywords:** Haemocyte, ABC transporters, Marine mussels, Glyphosate, AMPA, Multixenobiotic
44 resistance system
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Highlights

- *Mytilus galloprovincialis* haemocytes were treated in vitro with glyphosate (GLY), AMPA, and GLY+AMPA.
- GLY and AMPA increased MXR efflux activity and modulated *ABCB* expression
- The mixtures GLY+AMPA reduced efflux activity with *ABCB* down-regulation (at 1 µg/L GLY/AMPA).
- Modulation of lysosomal and immune related transcripts generally agree with known effects of the chemicals.
- Acting as chemosensitizers of the MXR system, GLY and AMPA may rise biological vulnerability of marine habitats.

1 Introduction

1
2 Among contaminants that may be relevant for environmental quality of coastal marine
3 areas, pesticides from agricultural and domestic activities are delivered by freshwaters
4 through run-off and leaching processes (Lupi et al., 2019). Herbicide formulations
5 containing glyphosate [N-(phosphonomethyl) glycine] as the active ingredient are among
6 the most employed pesticides worldwide, with an estimated use of about 600–750
7 thousand tons/year, which is predicted to raise up to 740–920 thousand tons/year by 2025
8 (Maggi et al., 2020). Glyphosate is a broad-spectrum, systemic, non-selective, and post-
9 emergence herbicide employed as a plant growth regulator. This compound inhibits the
10 enzyme 5-enolpyruvylshikimate-3-phosphate synthase that participates in the biosynthesis
11 of essential aromatic amino acids as phenylalanine, tyrosine, and tryptophan, hence
12 disrupting protein synthesis in bacteria, fungi, and plants (Schönbrunn et al., 2001;
13 Shehata et al., 2013; Shilo et al., 2016).

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Glyphosate is readily water soluble, and hydrolysis (> 30 days at pH 5 - 9) and photolysis (69 and 77 days, at pH 7 and 9, respectively) times reveal its relative persistence, that result in residence times in seawater of about 47 to 315 days (depending on light conditions, and within a 25 - 31°C temperature range) (Mercurio et al., 2014). Nevertheless, glyphosate can be degraded by bacteria into aminomethylphosphonic acid (AMPA) (Giesy et al., 2000). As a result, glyphosate and AMPA are frequently found together in the environment. In freshwaters, glyphosate concentrations range from 10 ng/L to about 1 mg/L (Brovini et al., 2021). Skeff et al. (2015) measured glyphosate and AMPA in ten estuaries along the German coasts of the Baltic Sea, showing variable average concentrations of 29-665 ng/L for glyphosate and 66-1445 ng/L for AMPA in most of the sampled rivers. Reports on seawater concentrations are restricted to some coastal sites of the Baltic Sea, where both glyphosate and AMPA have been detected at the low ng/L range (about 0.5 to 1.5 ng/L) (Wirth et al., 2021). Nevertheless, occurrence of glyphosate

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in the Lagoon of Venice (Italy) has been reported recently, with detected maximum concentrations between 260 and 7 ng/L for lagoon waters and suspended particulate matter, respectively, and 15 ng/g for sediment (Feltracco et al., 2022).

Several studies on glyphosate and AMPA highlighted a wide range of adverse effects in non-target aquatic species, from alteration of signal transduction pathways, increased oxidative stress, disruption of pro-apoptotic signaling, metabolism and reproduction in fish (Bernardi et al., 2022; Faria et al., 2021; Jia et al., 2022; Martins et al., 2021), to disruption of transcription for genes involved in apoptosis, immune response and cell signaling, stress responses, as well as embryotoxicity in aquatic invertebrates (Bringer et al., 2021; Iori et al., 2020; Milan et al., 2018). In marine bivalves, environmentally realistic concentrations of the compounds affected several haemocyte parameters in exposed animals (Matozzo et al., 2019a, 2019b, 2018a, 2018b).

This study investigates the effects of glyphosate, AMPA, and their mixture (the most environmentally realistic exposure scenario) on cytoprotective/detoxification mechanisms expressed in haemocytes of the Mediterranean mussel (*Mytilus galloprovincialis*). The circulating haemocytes of marine bivalves are vital immune effector cells, and are included in biomarker studies for their multiple functions, as they contribute to the physiological homeostasis, defense against pathogens, and to the stress response (Ladhar-Chaabouni and Hamza-Chaffai, 2016; Mao et al., 2020). In a previous study transcriptomic investigations with mussels revealed that ABC transporters may represent a main mechanism of defense/response to glyphosate exposure in mussels (Milan et al., 2018). Accordingly, in this study an *in vitro* assay was set to specifically assess transcriptional changes of the ABCB gene encoding the mussel ABC transporter P-glycoprotein in treated haemocytes along with the evaluation of global Multixenobiotic Resistance (MXR) efflux activity (Rasika Wathsala et al., 2018). To gain deeper insights into potential glyphosate and/or AMPA effects on mussel haemocytes, transcriptional changes of genes involved in

1 lysosomal and immune responses were further investigated, as these processes are
2 integral to haemocyte cell physiology, and have been addressed to as potential targets of
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5 glyphosate biological effects.
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10 **2 Methods**

11 *2.1 Chemicals*

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14 Rhodamine 123 (Rho123) was at the molecular biology grade (> 99% purity) and
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16 was purchased from Sigma Aldrich (Milan, Italy). The DirectZol kit was from Zymo
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18 Research (Freiburg, Germany). The iScript supermix and iTaq Universal master mix with
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20 ROX were from Biorad Laboratories (Milan, Italy). Any other reagent was from Sigma
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22 Aldrich (Milan, Italy).
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28 *2.2 Animal handling*

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30 Mediterranean mussels (*Mytilus galloprovincialis*) of commercial size (4–6 cm in
31
32 length) were obtained from a government certified mussel farm (Cooperativa Copr.al.mo,
33
34 Cesenatico, Italy). They were transferred to the laboratory in seawater tanks and
35
36 acclimated in aquaria containing 35-psu filtered seawater at 16 °C with continuous
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38 aeration (>90% oxygen saturation). During acclimatization, mussels were fed once a day
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40 with a commercial algal slurry (Koral, Xaqua).
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49 *2.3 Haemocyte preparation and treatments*

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52 Haemolymph was extracted from the posterior adductor muscle of different
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54 individuals using a sterile 1-mL syringe and was pooled to obtain the total volume suitable
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56 to perform each experiment. About 80 mussels for experimental trial have been employed
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58 (240 total mussels for the whole 3 experimental trials). For both MXR activity assay and
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1 RNA extraction, fresh pooled haemolymph was plated in 12-well plates (1 mL/well) and
2 settled for 1 h at 16°C in the dark. Cell attachment to the bottom of the well was checked
3
4 by microscopic inspection. The medium was then removed, and after 2 washings, replaced
5
6 with 1 mL artificial sea water (ASW) for control samples, or 1 mL ASW containing 0.1 µg/L,
7
8 or 1.0 µg/L glyphosate (GLY treatment), 0.1 µg/L, or 1.0 µg/L AMPA (AMPA treatment),
9
10 or 1.0 µg/L glyphosate (GLY treatment), 0.1 µg/L, or 1.0 µg/L AMPA (AMPA treatment),
11
12 and two mixtures (GLY+AMPA treatments) with 0.1 µg/L GLY + 0.1 µg/L AMPA, or 1.0
13
14 µg/L GLY + 1.0 µg/L AMPA, respectively. Tested concentrations were selected
15
16 considering previous laboratory studies with bivalves (Bringer et al., 2021; Matozzo et al.,
17
18 2018b), and reported seawater concentrations (Feltracco et al., 2022; Wirth et al., 2021).
19
20 Concentrations of the compounds in the tested media were checked by a validated
21
22 method based on separation of the derivatized compounds using 4-fluoro-7-nitro-2,1,3-
23
24 benzoxadiazole, by capillary electrophoresis and the detection through a light-emitting
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26 diode induced fluorescence detector (LEDIF) operating with the excitation wavelength at
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28 480 nm (fluorescence emission was at 515-760 nm) (Gotti et al. submitted to Journal of
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30 Chromatography A. *Reference will be updated upon ms acceptance*).
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36 All incubations were carried out at 16°C in the dark. Each treatment consisted of 3
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38 independent experiments. Within each experimental trial, 6 wells from the 12-well plates
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40 were employed for each treatment level, which were randomly pooled to give the 3
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42 replicates for each biological endpoint (N = 3).
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50 2.4 MXR activity assay

51 The assay was based on the analysis of cell-based transport using the model MXR
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53 fluorescent substrate rhodamine 123 (Rho123) (Smital et al., 2000). Given its physical
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55 properties, Rho123 is effectively extruded by ABC efflux pumps (Luckenbach et al., 2014).
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57 Therefore, cellular Rho123 content is a measure of transporter activity: a weak Rho123
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fluorescence signal corresponds to high activity of transporters, while a stronger fluorescence signal corresponds to lower transporter activity.

Assays were performed as reported previously (Franzellitti et al., 2016). Rho123 (2.5 μ M final concentration; stock solution prepared in DMSO, with solvent concentrations not exceeding 0.1% v/v in water) was added to treated and control wells to achieve a 90-min co-incubation period at 16 °C in the dark. The duration of exposure and Rho123 concentration were selected during preliminary experimental trials, which showed that these conditions did not significantly affect cell viability and provide stable and repeatable fluorescence readings (Franzellitti et al., 2019, 2016). At the end of the incubation period, samples have been processed according to Franzellitti et al. (2016) adapted for fluorescence measurements using a microplate reader system (Varioskan, Thermo Fisher; λ excitation = 485 nm; λ emission = 530 nm). Values were normalized to total protein content using the Qubit protein assay with the Qubit 2.0 system (Thermo Fisher) according to the manufacturer's instructions. Results were expressed as mean \pm SEM of the percentage of variation relative to controls.

2.5 *Quantitative real-time PCR analysis of mussel mRNA expressions*

Total RNA was immediately extracted from control and treated haemocytes using 1 mL of the Tri Reagent and the DirectZol kit according to the manufacturer's protocol. RNA concentration and quality were verified using the Qubit RNA assay (Thermo Fisher) and electrophoresis using a 1.2% agarose gel under denaturing conditions. First strand cDNA for each sample was synthesized from 500 ng total RNA using the iScript supermix following the manufacture's protocol.

ABCB mRNA expression was assessed by quantitative real-time PCR (qPCR) using primer pairs reported previously (Franzellitti and Fabbri, 2013). Primer pairs and

1 protocols employed for qPCR analyses of lysosomal and immune related transcripts are
2 as reported previously (Capolupo et al., 2018). Amplifications were detected with a
3
4 StepOne real time PCR system apparatus (Life Technologies, Milan, Italy) using a
5
6 standard “fast mode” thermal protocol. For each target mRNA, melting curve, and gel
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8 picture were analysed to verify the specificity of the amplified products and the absence of
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10 artifacts.
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14 A normalization factor, calculated using the geNorm software (Vandesompele et al.,
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16 2002) and based on the expression levels of the best performing reference transcripts in
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18 the analysed samples, was used for qPCR data normalization. The most stable reference
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20 gene products under the different experimental conditions tested were *18S rRNA* and
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22 *elongation factor 1 α* . Relative expression of target mRNAs in comparison with those of the
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24 reference gene products was calculated by a comparative C_T method (Schmittgen and
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26 Livak, 2008) Data were reported as mean \pm SEM of the normalized relative expression
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28 (fold change) with respect to controls.
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38 2.6 Statistical analysis

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40 Data from MXR efflux assay were analysed using non-parametric one-way ANOVA
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42 (Kruskal-Wallis test) followed by the Mann-Whitney U-test, after deviations from parametric
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44 ANOVA assumptions were observed in appropriate tests (Normality: Shapiro-Wilk’s test;
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46 equal variance: F-test). These statistical analyses were performed using the
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48 GraphPadPrism 9 software (GraphPad Inc.). qPCR data were evaluated with the REST
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50 software (Pfaffl et al., 2002) that uses a randomisation test with a pairwise reallocation to
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52 assess the statistical significance of the differences in expression between each treatment-
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54 exposed group and the controls. Further comparisons between pair of treatments were
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1 performed using the Mann-Whitney U-test. In all approaches, $p < 0.05$ was set as the
2 threshold level of statistical significance.
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5 All the biological endpoints assessed were further submitted to permutation
6 multivariate analysis using the PRIMER v6 software (Anderson et al., 2008). Log-
7 transformed MXR efflux assay data and fold change variations of the target transcripts
8 were used to calculate similarity matrices based on the Euclidean distance (999
9 permutations). From this similarity matrices, a nMDS analysis (non-metric multi-
10 dimensional scaling), and analysis of similarity (one-way ANOSIM), were carried out. One-
11 way ANOSIM was selected as non-parametric statistical method to analyze the treatment
12 grouping significance.
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28 **3 Results**

29 *3.1 Effects of GLY, AMPA, and the mixture GLY+AMPA on MXR efflux activity and* 30 31 32 33 34 *ABCB mRNA expressions* 35

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37 MXR transport activity was assessed following a 90-min treatment with GLY, AMPA,
38 or the mixture GLY+AMPA (Fig 1A). A concomitant assessment of *ABCB* expression
39 under the same treatment conditions was also performed (Fig 1B). Both GLY and AMPA
40 treatments induced a significant reduction of Rho123 accumulation at 0.1 and 1 $\mu\text{g/L}$ (Fig
41 1A), indicating increased efflux activity. The *ABCB* transcript was significantly down-
42 regulated at 0.1 $\mu\text{g/L}$ GLY and up-regulated at 1 $\mu\text{g/L}$, while no significant changes
43 occurred in cells treated with AMPA at both concentrations (Fig 1B). The mixture
44 GLY+AMPA induced a significant increase of Rho123 accumulation (Fig 1A), indicating
45 reduced efflux activity, with a concomitant *ABCB* down-regulation at 1 $\mu\text{g/L}$ either GLY or
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3 **3.2 Effects of GLY, AMPA, and the mixture GLY+AMPA on haemocyte gene**
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5 *transcription*

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8 The effects of GLY, AMPA, or the mixture GLY+AMPA (0.1 or 1 µg/L) on transcription
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10 of 6 genes involved in lysosomal and immune responses was evaluated in haemocytes
11
12 treated in vitro (Fig 2). Full gene names are reported in caption to Fig 2. GLY induced
13
14 significant down-regulation of all transcripts encoding lysosomal enzymes at both tested
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16 concentrations (*HEX* and *GUSB*) or at 0.1 µg/L (*CTSL*) (Fig 2A-C). Both AMPA and the
17
18 mixture GLY+AMPA induced a significant down-regulation of *HEX* and *GUSB* (1 µg/L),
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20 while *CTSL* resulted either up- (0.1 µg/L) or down- (1 µg/L) regulated (Fig 2A-C).
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25 As to transcripts involved in the immune response, *GLY* induced significant down-
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27 regulation of *LYS* and up-regulation of *MYTCc* at both tested concentrations, while
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29 expression levels of *MYTLb* were unchanged compared to control samples (Fig 2D-F).
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31 AMPA induced down-regulation of *LYS* at 0.1 µg/L, and of *MYTLb* and *MYTc* at 1 µg/L
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33 (Fig 2D-F). The mixture GLY+AMPA significantly affected only *MYTLb* transcription (up-
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35 regulation at 0.1 µg/L) (Fig 2D-F).
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43 **3.3 Multivariate statistical analyses**

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45 nMDS analysis based on the whole dataset of the biological endpoints showed that
46
47 samples clustered according to the different treatments and concentrations (Fig 3A). GLY
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49 treatments are well separated from the other groups. GLY+AMPA treatments clustered
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51 close to controls, followed by AMPA treatments (Fig 3A). To determine whether this
52
53 grouping was meaningful, a non-parametric analysis of similarity (ANOSIM) testing all
54
55 sample groups independently (9 groups: 3 treatments, 3 concentrations, including controls
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57 at 0 µg/L either GLY or AMPA) on the whole dataset and on the single biological endpoints
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1 was performed (Fig 3B). R values close to 1 indicate a dissimilarity between pairs groups;
2 in contrast, when the R value is near 0, there is no significant difference between pairs of
3 groups. GLY treatment resulted in significant dissimilarity of the overall biological response
4 and of selected endpoints (MXR efflux activity, *ABCB*, *GUSB*, *MYTCC*, *MYTLb*) in relation
5 to AMPA or GLY+AMPA treatments ($p < 0.05$; Fig 3B). Comparison of AMPA and
6 GLY+AMPA treatments produced the lowest and less significant R values, hence
7 indicating no significant difference between these groups (Fig 3B).
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21 **4 Discussion**

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23 Induction of ABC transporter activity has been related to glyphosate resistance in
24 plants (Amrhein and Martinoia, 2021; Ge et al., 2014; Nol et al., 2012), as well as in
25 zebrafish (Fernanda Moreira et al., 2019; Moraes et al., 2020) and in the Atlantic salmon
26 (Søfteland and Olsvik, 2022). In agreement, in this study the mussel MXR efflux activity
27 was increased by either glyphosate or its metabolite AMPA in haemocytes treated *in vitro*.
28 A concomitant over-expression of a *ABCB* transcript encoding the P-glycoprotein, the best
29 investigated mussel ABC transporter, was observed. These findings corroborate a
30 previous investigation reporting the transcriptional modulation of ABC transporters in
31 digestive glands of mussels exposed *in vivo* to glyphosate (Milan et al., 2018). Taken
32 together, the data suggest that modification of ABC transporter capacities either at the
33 transcriptional or at the functional level may represent a generalized mechanism to
34 improve defense and response to glyphosate, and our data show that this may hold true
35 also for AMPA. The mixture GLY+AMPA apparently reduced MXR efflux activity, as
36 Rho123 accumulation resulted increased compared to control cells. This may occur
37 because glyphosate and/or AMPA may be preferentially transported in place of Rho123,
38 that is accumulated within the cell, or, alternatively, because overall pollutant cell load may
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lead to chemosensitization. Indeed, it is known that pollutants (i.e., pesticides, fragrances, pharmaceuticals and polyaromatic hydrocarbons) acting as chemosensitizers exert their action by saturating the transport system leading to its inhibition at high concentrations (Smital and Kurelec, 1998). *ABCB* down-regulation observed at the highest GLY/AMPA concentrations tested in the mixture may be a feedback effect related to efflux activity inhibition that corroborates the latter hypothesis, although the action of the compounds as transcriptional regulators of *ABCB* may not be excluded, as previously reported for other pollutants acting as MXR substrates (Franzellitti et al., 2016).

Lysosomes have been shown as potential targets for glyphosate effects in a zebrafish cell line (Goulart et al., 2015). In agreement, in this study differential regulation of transcripts encoding lysosomal enzymes was observed by GLY and by AMPA and in haemocyte cells treated with the mixture GLY+AMPA. Hexosaminidase (HEX) is a lysosome enzyme employed as a marker for changes in lysosome membrane stability, hence *HEX* down-regulation at all tested treatments suggest a potential decrease of lysosome membrane stability (Izagirre and Marigómez, 2009). β -glucuronidase (*GUSB*) is related to lysosomal structural changes including lysosome size (Izagirre et al., 2009; Marigómez et al., 2005). *GUSB* down-regulation may indicate a reduction of lysosome structural integrity. Finally, Cathepsin L (*CTSL*) is involved in intracellular protein catabolism, and it is a component of the autophagic pathway (Xu et al., 2021). Either *CTSL* up- or down- regulation may be a signature for alteration of the lysosomal system, as both increased or depleted *CTSL* enzyme activity was observed in *Unio tumidus* haemocytes after *in vivo* exposure to several pollutants, including Roundup, the commercial formulation containing glyphosate (Khoma et al., 2021).

Up-regulation of immune gene transcription was previously observed in digestive glands of mussels exposed for 21 days to 100 μ g/L GLY, 100 μ g/L AMPA, or a mixture of 100 μ g/L GLY + 100 μ g/L AMPA (Iori et al., 2020) and related to perturbations of digestive

1 gland microbiota composition. In this study, we observed a limited transcriptional
2 modulation of immune genes in haemocytes, with either up- or down- regulation,
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4 apparently suggesting a lack of host immune system activation. Nevertheless, it is worth
5
6 noting that the *in vitro* exposure scheme employed in this study did not allow to test for the
7
8 interaction between GLY/AMPA, haemolymph microbiota and the haemocyte immune
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10 response. *In vivo* studies comparing microbiota responses to contaminants in different
11
12 tissues would better address this issue. Mussel haemolymph and digestive gland have
13
14 widely different microbiota structure reflecting conditions controlled by external
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16 (environmentally-driven, haemolymph) or internal (related to metabolic and detoxification
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18 processes, digestive glands) selective pressures (Musella et al., 2020). Therefore, it may
19
20 be reasonable that glyphosate/AMPA impact on microbiota (and vice versa) and related
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22 downstream mussel responses may peak in digestive glands, where chemical
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24 bioaccumulations and detoxifications occur (Faggio et al., 2018), along with enrichment of
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26 microbiota components supporting these functions (Musella et al., 2020).
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37 **5 Conclusions**

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40 Radar plots that summarize changes of the biological endpoints under the different
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42 treatment groups are reported in Fig 4. Together with multivariate analyses, they suggest
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44 that haemocyte treatment with AMPA and a mixture GLY+AMPA have a limited global
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46 impact compared to GLY treatment. As to AMPA, this finding agrees with a large body of
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48 experimental evidence showing that the metabolite is less toxic than the parental
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50 compound (Wang et al., 2022). In the mixture GLY+AMPA, cumulative or interactive
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52 effects between the two compounds should be hypothesized. Under the hypothesis that in
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54 the mixture GLY+AMPA the MXR efflux activity is significantly reduced due to a putative
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56 cumulative chemosensitizer action, both compounds may not be effectively cleared from
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1 the intracellular environment, leading to increased cytotoxicity and reduced magnitude of
2 the biological responses. Furthermore, AMPA and the mixture GLY+AMPA seem to have
3 a greater impact on lysosomal responses, specifically on *CTSL* transcription, which is a
4 proxy for altered protein catabolism.
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9 The proved occurrence of glyphosate and AMPA in coastal marine ecosystems
10 (Feltracco et al., 2022; Wirth et al., 2021) is fostering the research to disclose their
11 potential detrimental effects on marine fauna. On the whole, results of this study suggest
12 that, by their ability to cumulatively act as chemosensitizers lowering the cytoprotective
13 role of the MXR system, and to exert interactive effects on haemocyte cell parameters,
14 GLY and AMPA co-occurrence even at (relatively low) environmental concentrations
15 should be addressed as a factor of concern for the biological vulnerability of coastal
16 marine habitats.
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37 Bologna, Italy).
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Figure legends

1
2 **Fig 1. Effects of glyphosate (GLY), its metabolite AMPA, and their mixture**
3 **(GLY+AMPA) on the MXR system of *M. galloprovincialis* haemocytes.** Cells were
4
5 treated in vitro with 0.1 µg/L and 1.0 µg/L glyphosate (GLY treatment), 0.1 µg/L and 1.0
6
7 µg/L AMPA (AMPA treatment), and two mixtures (GLY+AMPA treatment) with 0.1 µg/L
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9 GLY + 0.1 µg/L AMPA, or 1.0 µg/L GLY + 1.0 µg/L AMPA, respectively. (A) Rho123
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11 accumulation following a 100-min treatment. Data are expressed as mean ± SEM (N = 3)
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13 of the variation vs controls (untreated cells) (*p < 0.05). (B) *ABCB* expression changes.
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15 Data are expressed as mean ± SEM (N = 3) of the relative variation (fold change) with
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17 respect to controls (*p < 0.05). **Colored figure is intended only for the online and PDF**
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19 **version.**
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29 **Fig 2. Effects of glyphosate (GLY), its metabolite AMPA, and their mixture**
30 **(GLY+AMPA) on gene transcription in *M. galloprovincialis* haemocytes.** Cells were
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32 treated in vitro with 0.1 µg/L and 1.0 µg/L glyphosate (GLY treatment), 0.1 µg/L and 1.0
33
34 µg/L AMPA (AMPA treatment), and two mixtures (GLY+AMPA treatment) with 0.1 µg/L
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36 GLY + 0.1 µg/L AMPA, or 1.0 µg/L GLY + 1.0 µg/L AMPA, respectively. Relative
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38 expression of transcripts involved in lysosomal system: (A) hexosaminidase, *HEX*, (B)
39
40 cathepsin L, *CTSL*, (C) β-glucuronidase, *GUSB*, and immune responses: (D) lysozyme,
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42 *LYS*, (E) mytilin b, *MYTLb*, (F) myticin c, *MYTCc*. Data are reported as mean ± SEM of 3
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44 experiments carried out in 6-multiwell plates (3 replicate wells for each sample) (N = 3).
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46 Values represent the relative variation (fold change) vs controls (untreated cells) (*p <
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48 0.05). **Colored figure is intended only for the online and PDF version**
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58 **Fig 3. Multivariate analyses.** (A) Nonmetric multidimensional scaling (nMDS) biplot of
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60 multivariate patterns of the whole biological endpoints. 95% confidence ellipses for the
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1 different treatment groups and tested concentrations are reported. (B) Non-parametric
2 analysis of similarity (ANOSIM) testing all sample groups (9 groups: 3 treatments, three
3 concentrations, including controls at 0 µg/L either GLY or AMPA) on the whole dataset and
4 on the single biological endpoints. The graph reports the R values between pairs of group
5 comparisons and their levels of significance (**p ≤ 0.01, *p ≤ 0.05). See Results Section
6 for more details. **Colored figure is intended only for the online and PDF version.**
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17 **Fig 4. Radar plots summarizing the effects of GLY, AMPA and the mixture**
18 **GLY+AMPA in *M. galloprovincialis* haemocytes.** For each biological endpoint,
19 concentration-related variation is expressed by the Area Under the Curve (AUC) according
20 to Rasika Wathsala et al. (2018). **Colored figure is intended only for the online and**
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FIG 1

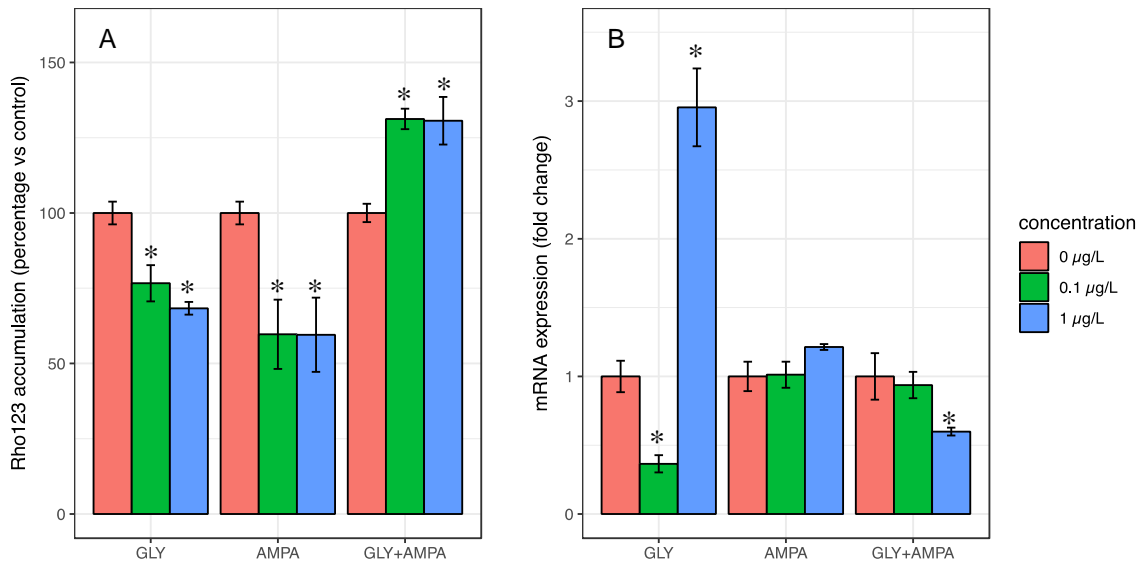


FIG 2

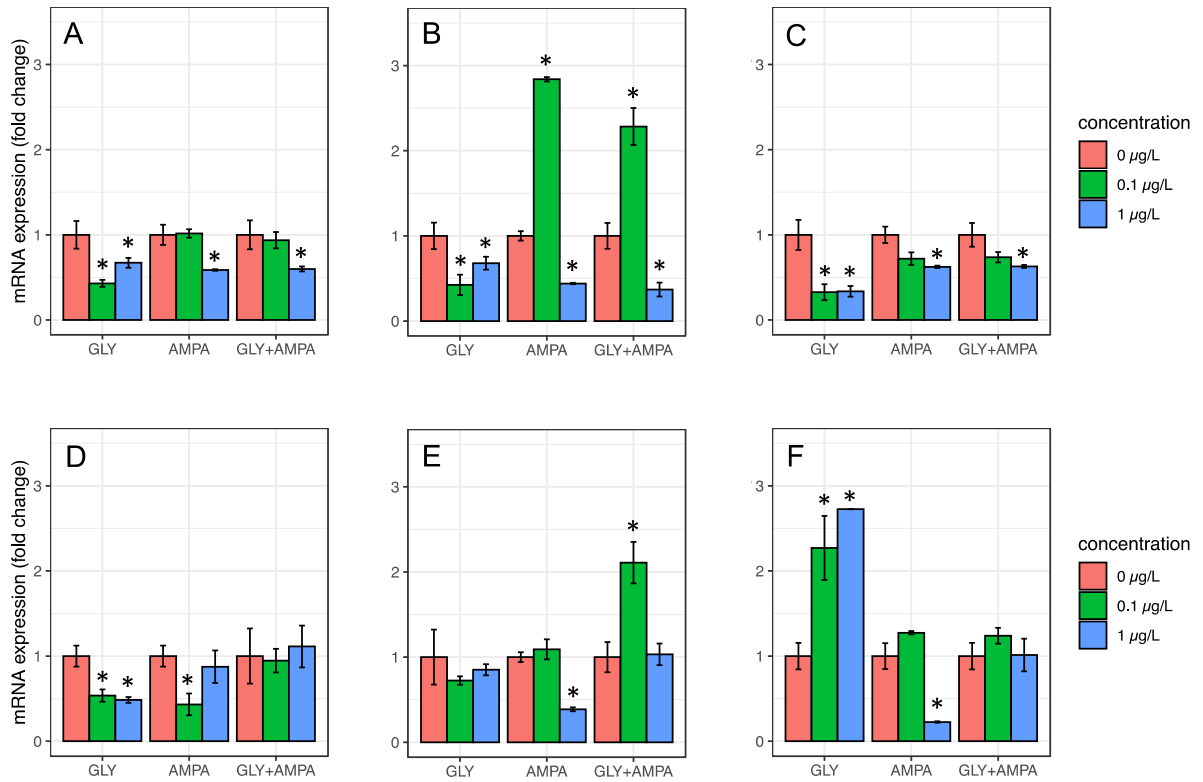
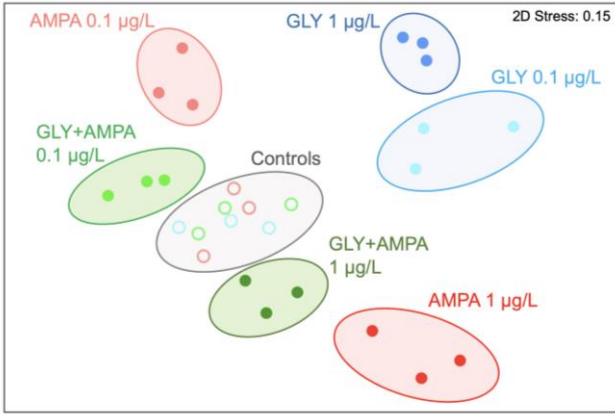


FIG 3

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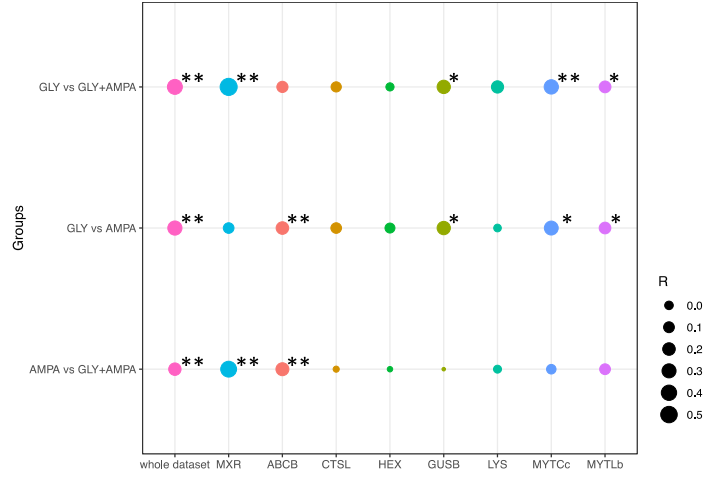
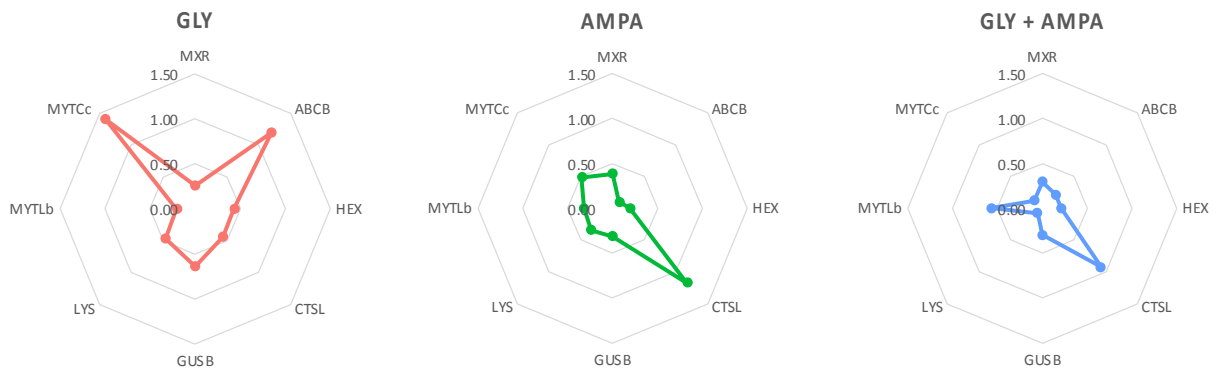


FIG 4



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