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Do extreme salinity fluctuations exacerbate AMPA toxicity? Experimental evidence and hazard characterization in Mediterranean mussels

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ABSTRACT

Climate change may exacerbate extreme weather events, leading to severe salinity fluctuations in coastal waters and enhancing the mobility of land-based pollutants such as aminomethylphosphonic acid (AMPA), the main breakdown product of glyphosate. Although the individual effects of salinity stress and AMPA are increasingly characterized, their combined impact on marine organisms is still poorly investigated.

This study aimed to assess, for the first time, the effects of different salinity scenarios (35, 25, 15) on the toxicity of environmentally realistic concentrations of AMPA (0.5 μg/L) in *Mytilus galloprovincialis*. A wide panel of cholinergic functions, immunological responses, antioxidant defences and oxidative damage, peroxisomal proliferation, and energy metabolism was evaluated after 21 days of exposure, and the results elaborated using weighted criteria to provide hazard classification based on magnitude and toxicological relevance of observed effects. Results indicated that AMPA exposure significantly influenced cholinergic responses, detoxification mechanisms and antioxidant defences. Beyond the direct effects, salinity modulated AMPA toxicity, either exacerbating or counteracting specific effects on immune function, oxidative balance, and metabolic homeostasis, with tissue-specific patterns. The Weight of Evidence approach assigned a higher hazard classification to combined stressor scenarios, further corroborating the importance of exploring interactive toxicological effects. Overall, this work provided a novel integrative toxicological profiling of AMPA under climate change-driven salinity stress, emphasizing the need to consider the interactions between multiple co-occurring stressors in environmental risk assessment frameworks.

1. Introduction

Climate change is an urgent global threat to aquatic ecosystems, triggering cascading impacts on biodiversity, ecosystem services, and human well-being (Cooley et al., 2022; IPCC et al., 2023). One of the most critical manifestations of climate change is the intensification of extreme events, such as heavy rainfall and flooding, which can result in massive freshwater inflows into coastal areas, leading to rapid but progressive and marked decreases in salinity. While freshwater inputs can reach coastal waters within short time lags (≈1 day), low-salinity conditions typically develop over a few days and persist for weeks, driven by hydrodynamic factors, such as plume dynamics and mixing processes (Durack, 2015; Poppeschi et al., 2021; ARPAE, 2023; Sabater et al., 2023; Behera, 2024). Such conditions have been reported following extreme rainfall events in coastal systems, including south-western Australia (Malan et al., 2024), the northwest Arabian Gulf (Alosairi et al., 2019), the Elbe Estuary, where salinity dropped below 3 for over a week (Voynova et al., 2017), and the Bay of Brest, where low-salinity conditions persisted for up to two weeks (Poppeschi et al., 2021).

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Such salinity fluctuations in estuarine and marine ecosystems might impose significant osmotic stress on aquatic biota, particularly on sessile invertebrates such as bivalves, which cannot escape harsh environmental conditions. To cope with salinity fluctuations, bivalves rely on cellular volume regulation mechanisms, adjustments in inorganic ions and organic osmolytes, particularly free amino acids such as proline, serine, glutamate, glycine, taurine, and alanine, which preserve protein function and stability (Silva and Wright, 1994; Pourmozaffar et al., 2020; Tan et al., 2023). Salinity stress has also been observed to induce a broad spectrum of molecular, biochemical and cellular effects in marine bivalves. Ionic homeostasis is crucial for immunocyte functioning, with different effects observed across species. Among these, reduced lysosomal membrane stability was reported in *Ostrea edulis* at low salinity (16 and 19), decreased phagocytosis in *Crassostrea gigas* and *Pinctada imbricata* at salinity 15 and 25, respectively, altered haemocyte counts in *Ruditapes philippinarum* and *Chamelea gallina* at hyposaline (28) conditions, and reduced cytotoxicity in *Mytilus galloprovincialis* at salinity 25 (Matozzo and Marin, 2011 and references therein). Moreover, salinity stress has been described to modulate cholinergic activity (Pfeifer et al., 2005), and redox homeostasis, leading to pro-oxidant conditions through increased production of reactive oxygen species (ROS) and/or modulation of antioxidant defences. Such imbalances were shown to compromise cellular integrity and increase oxidative damage to macromolecules in *M. galloprovincialis* (Freitas et al., 2017) and lipid peroxidation levels in *R. decussata, R. corrugata, R. philippinarum, Crassostrea angulata* and *M. galloprovincialis* (Carregosa et al., 2014b; Moreira et al., 2016; Freitas et al., 2017).

Under realistic environmental scenarios, marine organisms face multiple co-occurring stressors that synergistically influence stress-response mechanisms and tolerance. Indeed, runoff events related to rainfall and floods might enhance the transport of land-based chemical contaminants from terrestrial to aquatic environments, including several pesticides and herbicides such as glyphosate and its main breakdown product, aminomethylphosphonic acid (AMPA) (Struger et al., 2015; Lefrancq et al., 2017). AMPA is receiving attention due to its increasingly documented environmental occurrence in both freshwater and marine ecosystems at variable concentrations depending on the matrix, environmental and weather conditions and proximity to sources (Battaglin et al., 2014; Skeff et al., 2015; Wang et al., 2016; Grandcoin et al., 2017; Klátyik et al., 2024). After rainfall events, levels of AMPA in runoff water have been reported to increase up to 47 μg/L in the outlet of a vineyard in France (Lefrancq et al., 2017), while in riverine waters of the Veneto region (Italy), mean values of 180 ng/L and peaks up to 770 ng/L were detected (Masiol et al., 2018). In estuarine environments, concentrations ranged from 4156 to 45 ng/L in ten German estuaries (Skeff et al., 2015). A marked decrease in AMPA concentrations along the freshwater-marine gradient has been reported in the Warnow Estuary (Baltic Sea), from 2633 ng L^{-1} at the outlet of a wastewater treatment plant to less than 8 ng L^{-1} in coastal waters, likely reflecting dilution processes (Wirth et al., 2021). Using more sensitive methods, AMPA was still detectable in coastal waters at low concentrations (0.6–1.42 ng L^{-1}), while remaining below detection limits at offshore stations (0.22 ng L^{-1}). A similar decreasing trend along the freshwater-marine gradient was observed in the Elbe estuary, with concentrations of a few μg/L in coastal sites declining to ~1 μg/L in the Wadden Sea (Pupke et al., 2016).

Due to strong C-P chemical bonds, AMPA is characterized by a limited natural degradation and elevated persistence in aquatic environments, thus representing a long-lasting contaminant in both freshwater and marine environments. Detrimental and toxic effects of AMPA have been documented in non-target aquatic organisms with consequences that, according to a recent but growing body of evidence, would be more severe than those exhibited by its parent compound glyphosate (Bento et al., 2016; Grandcoin et al., 2017; Tresnakova et al., 2021; Barreto et al., 2023; Nardi et al., 2025).

Comprehensive evidence of AMPA capability to disrupt multiple cellular processes was recently shown in *M. galloprovincialis,* including neuro-immune function, DNA integrity, xenobiotic metabolism, lipid and energy homeostasis (Nardi et al., 2025). Oxidative stress and alterations of haemocyte parameters have also been reported in mussels (Matozzo et al., 2018, 2019; Tresnakova et al., 2021), while mortality, developmental abnormalities and oxidative stress have been shown in early life stages of the catfish *Steindachneridion melanodermatum* (Barreto et al., 2023).

Given the increased mobility of AMPA associated with heavy rainfalls, a comprehensive characterization of its toxicological profile under different salinity scenarios would be of great importance for coastal species potentially co-exposed to both these stressors during extreme flooding events. In this respect, the biological effects of an environmentally realistic concentration of AMPA were evaluated under hyposalinity regimes, using *M. galloprovincialis* as a model species given its sensitivity to contaminants, wide distribution in coastal areas, ecological importance and significant economic value (Kristan et al., 2014; Musella et al., 2020). A comprehensive panel of 42 biochemical and cellular responses, recognized for their responsiveness to chemical and osmotic stressors, was analysed in mussels' haemocytes, gills and digestive glands, including immunocytes populations and functionality, antioxidant defences and oxidative damages, cholinergic function, detoxification mechanisms, peroxisomal proliferation and lipid homeostasis, energy metabolism; such a wide and heterogeneous dataset was thus elaborated through a Weight of Evidence (WOE) approach, to provide a quantitative hazard profiling of tested scenarios based on the number, magnitude and toxicological relevance of observed alterations. The overall results were expected to address a critical yet virtually unexplored gap, offering novel insights towards more realistic ecotoxicological risk assessment of AMPA under a climate change scenario.

2. Material and methods

2.1. Animal collection and experimental plan

Mediterranean mussels *Mytilus galloprovincialis* (5 ± 0.3 cm shell length) were obtained from a mussel farm located approximately 2 nautical miles from the Conero Riviera shores (central Adriatic Sea, Italy) in August 2024. Once collected, 240 organisms were randomly distributed among 12 tanks (15 L each) and maintained in artificial seawater at seasonal environmental conditions (24 °C, salinity 35 and pH 8.2) for one week of acclimation. Then, salinity was gradually adjusted over the next 10 days at a rate of 1 and 2 units per 24 h until reaching target salinities of 25 (4 tanks) and 15 (4 tanks), respectively. This gradual adjustment was adopted to reproduce progressive salinity changes occurring in coastal marine environments following freshwater inflow events (Alosairi et al., 2019; Poppeschi et al., 2021; Malan et al., 2024), while avoiding acute osmotic shock. During this phase, organisms were fed daily with a commercial microalgae mixture (Easy Booster Nano, easy reefs®, composed by 33% of *Isochrysis*, 31% *Nannochloropsis*, 18% *Tetraselmis*, and 18% *Phaeodactylum*).

At the end of acclimation, tanks were randomly assigned to various treatments resulting from the combination of two AMPA concentrations (0 and 0.5 μg/L) and three salinity scenarios (35, 25 and 15). The AMPA concentration of 0.5 μg/L was selected as a high yet environmentally realistic level for estuarine and coastal systems influenced by riverine inputs (Skeff et al., 2015; Pupke et al., 2016; Wirth et al., 2021) and is consistent with a previous study from our group comparing glyphosate and AMPA effects in *M. galloprovincialis* (Nardi et al., 2025). Regarding salinity, levels of 25 and 15 were selected to represent moderate and extreme hyposalinity, mimicking freshwater inflow events in coastal systems and the resulting drop in salinity (Poppeschi et al., 2021; ARPAE, 2023). Thus, salinity 25 represents a severe yet realistic condition observed in the Adriatic, whereas 15 reflects an extreme scenario in shallow or semi-enclosed systems with high freshwater input and limited mixing. Accordingly, the following treatments (each performed in duplicate) were tested: SAL 35 (control), AMPA 35, SAL 25, AMPA 25, SAL 15, AMPA 15. During the exposure, the feeding regime was the same as the acclimation phase; the exposure media was completely changed, and AMPA was re-dosed every other day. A stock solution of AMPA (3000 mg/L) was prepared by dissolving the analytical standard (Sigma-Aldrich) in ultrapure water. This was further diluted to obtain an intermediate stock solution (300 mg/L), which was stored at 4 °C throughout the experiments: from this, a working solution (15 mg/L) was freshly prepared before each dosing. While AMPA concentration was not analysed throughout the experiment, a preliminary verification test was conducted in the exposure medium under the same setup (including microalgae as food but without mussels) to assess AMPA stability and consistency with nominal levels, following ISO 21458:2008. Measured concentrations ranged between 0.49 and 0.53 μg/L at 10 min after spiking and 0.47-0.50 μg/L after 48 h, remaining within 95-110% of the nominal concentration (0.5 μg/L).

After 21 days of exposure, organisms were collected from each experimental condition, and tissues were dissected for analyses. Haemolymph from 12 organisms was withdrawn (4 pools, each constituted by 3 organisms' haemolymph), partially used for *in vivo* analyses, and partially fixed in Carnoy's solution for micronuclei frequency analysis. For biochemical and cellular analyses, digestive glands, gills, and haemolymph from 24 organisms (12 per duplicate tank) were dissected and pooled into 8 samples (each containing tissues from 3 organisms), rapidly frozen in liquid nitrogen, and stored at −80 °C. Digestive glands from an additional 4 organisms were dissected, rapidly frozen in liquid nitrogen, and maintained at −80 °C until histochemical analyses.

2.2. Biological analyses

Standardized protocols were used to analyse the following biomarkers in mussel tissues, as further detailed in Supplementary Materials (SM): immunocytes composition and responses (lysosomal membrane stability, granulocytes/hyalinocytes ratio, phagocytosis activity and micronuclei frequency); esterases activity (acetylcholinesterase activity in haemolymph, gills and digestive glands; carboxylesterase activity in digestive glands and gills), antioxidant defences (catalase, superoxide dismutase, total glutathione peroxidases, glutathione S-transferase activities, glutathione reductase, GSH/GSSG ratio), total oxyradical scavenging capacity toward peroxyl and hydroxyl radicals in digestive glands and gills; lipid and proteins oxidative damages in digestive glands and gills (TBARS assay and proteins carbonylation); lipid homeostasis (acyl-CoA oxidase activity, neutral lipids and lipofuscin content) in the digestive glands; electron transport system activity, glycogen and proline content in digestive glands and gills.

2.3. Statistical analyses and Weight of Evidence elaboration

All statistical analyses were performed in RStudio (version 2024.12.1 + 563). Given the multivariate nature of the dataset, statistical inference combined multivariate and univariate approaches. Multivariate analyses were conducted using the *vegan* package. Principal Coordinates Analysis (PCoA) was performed using classical multidimensional scaling (*cmdscale*). Univariate analyses (two-way ANOVA, assumption tests, and post hoc comparisons) were performed using the *car*, *emmeans*, *multcomp*, and *multcompView* packages. Data handling and visualization were carried out using *tidyverse* and *ggplot2*, with additional graphical elements implemented using *ggpattern*, *cowplot*, and *gridExtra*.

Biomarker data were standardized (z-scores) prior to multivariate analysis, and a Euclidean distance matrix was computed. The effects of AMPA exposure (no AMPA vs. 0.5 μg/L AMPA), salinity (35, 25, 15), and their interaction on the overall biomarker profile were assessed using permutational multivariate analysis of variance (PERMANOVA; *adonis2*, 9999 permutations) with sequential (Type I) sums of squares. Homogeneity of multivariate dispersion among treatment groups was evaluated using the *betadisper* procedure followed by permutation testing. Patterns of multivariate separation were visualized using PCoA based on the same Euclidean distance matrix used for PERMANOVA. To identify the biomarkers most strongly associated with the ordination structure, vector fitting was performed using envfit (9999 permutations), retaining variables with $p < 0.05$. The full results of the envfit analysis are provided in Supplementary Materials (Table SM1).

Univariate two-way ANOVA was applied to each biomarker. Data were tested for normality (Shapiro–Wilk) and homogeneity of variances (Levene's test), and Box–Cox transformation was applied when needed. The effects of treatment (0 and 0.5 μg/L AMPA), salinity (35, 25, 15), and their interaction were assessed. Tukey HSD post hoc tests were used to evaluate differences among salinity levels within each treatment and between AMPA conditions at each salinity level (indicated in figures by letters and asterisks).

Finally, a quantitative Weight of Evidence (WOE) approach was applied to integrate the experimental results and derive synthetic hazard quotients (HQs) for each experimental condition. This approach is based on the elaboration and integration of data according to weights reflecting the toxicological relevance of measured biological endpoints, statistical significance, and the magnitude of observed variations relative to specific thresholds. The resulting HQs are then assigned to one of five classes of hazard, from “Absent” to “Severe”. Details, assumptions and whole calculation are described in previously published studies (Nardi et al., 2025; Regoli et al., 2019; Gonçalves et al., 2023) and summarized in SM (Fig. SM1).

3. Results

3.1. Multivariate analysis of biomarkers profile

The multivariate structure of the biomarker dataset was analysed through PERMANOVA to assess the effects of AMPA exposure, salinity, and their interaction on the overall biological response profile. Significant multivariate effects were detected for all factors, with a strong contribution of the AMPA and salinity interaction to the overall variability in biomarker patterns ($F_{(2,12)} = 3.62$, $R^2 = 0.211$, $p < 0.001$). Although main effects should not be interpreted independently with a significant interaction, both salinity ($F_{(2,12)} = 5.94$, $R^2 = 0.346$, $p < 0.001$) and AMPA exposure ($F_{(1,12)} = 3.26$, $R^2 = 0.095$, $p < 0.01$) significantly contributed to overall variance. Homogeneity of multivariate dispersion was confirmed (betadisper; $p = 0.926$).

Multivariate patterns were further explored using PCoA conducted on the same matrix used for PERMANOVA (Fig. 1). The two-dimensional ordination, explaining ~45% of total variance, showed a trend of clustering among different salinity treatments and a marked separation between AMPA-exposed and non-exposed organisms. This pattern was mainly highlighted along Axis 1 (29.6%), suggesting salinity as the primary driver of multivariate variability and indicating a marked shift in biomarker profiles under hyposaline conditions. Axis 2 (14.7%) further differentiated treatments and highlighted the interaction between AMPA exposure and salinity, supporting the non-additive effects observed in PERMANOVA. In particular, AMPA-exposed samples diverged from controls at salinities 35 and 15 (with opposite trends), whereas partial overlap was observed at salinity 25. Based on significance and r^2 values, Axis 1 separation was mainly driven by GH, proline content in gills, neutral lipids, AChE in haemolymph, ACOX and ETS in gills, and CEs (4NPB), whereas Axis 2 was mainly influenced by LPO, AChE and the GSH/GSSG ratio in gills, together with CAT in the digestive gland (Tab. SM1).

3.2. Acetylcholinesterase and carboxylesterases activity

The activity of acetylcholinesterase (AChE) was measured in haemolymph, gills and digestive glands (Fig. 2A–C, Table SM2 in SM). In

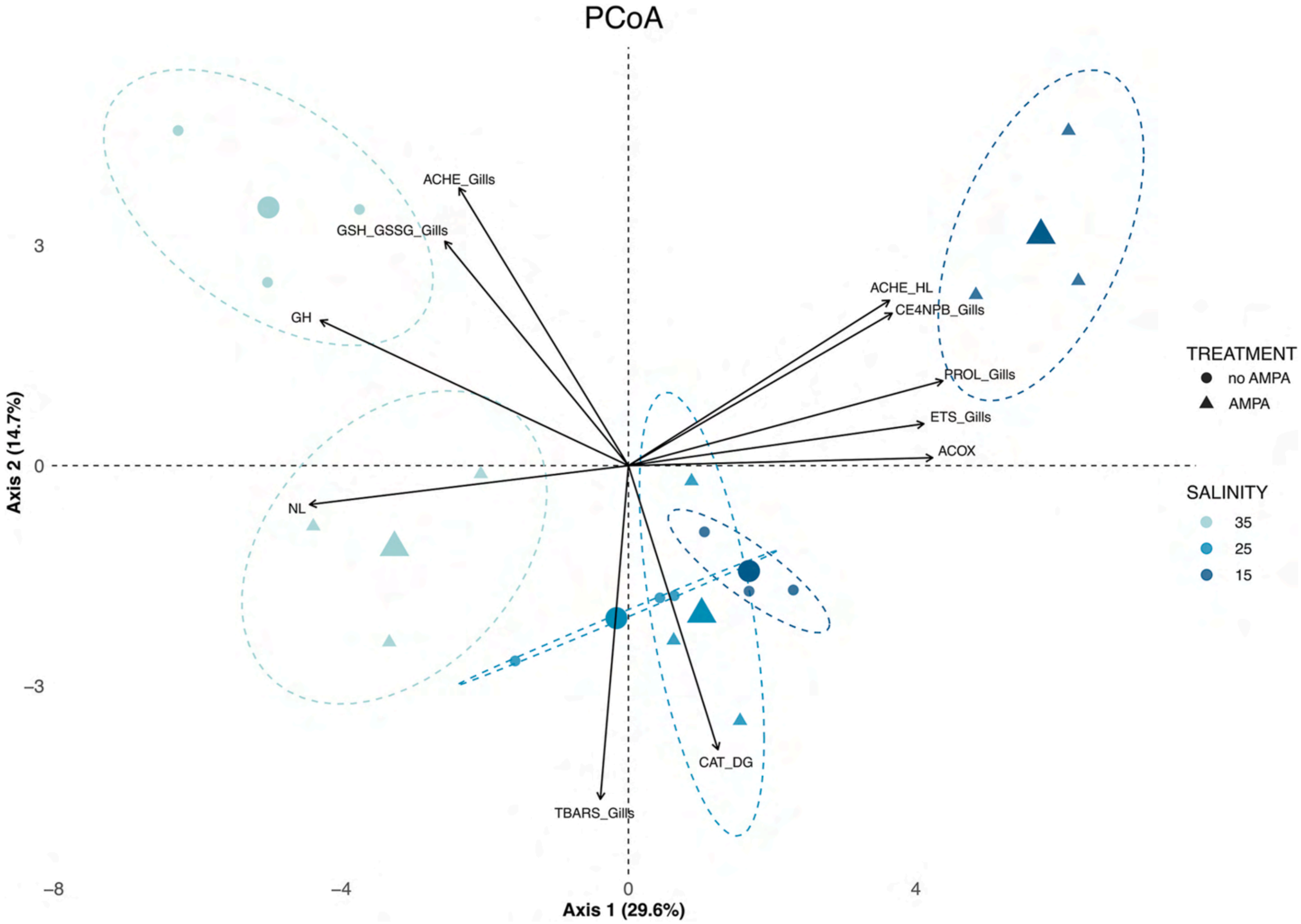


Fig. 1. Principal Coordinates Analysis (PCoA) of biomarker profiles. Two-dimensional representation of PCoA results for the experimental treatments, with symbols indicating AMPA treatments and colours representing salinity levels. Smaller symbols represent individual observations, while larger symbols indicate group centroids. Ellipses represent 95% confidence regions around group centroids. Arrows correspond to biological variables significantly associated with the ordination (envfit, $p < 0.05$, $r^2 > 0.4$), with their direction and length reflecting the strength and direction of their correlation with the multivariate pattern.

haemolymph, the activity significantly increased at the lowest salinity, regardless of AMPA presence (Fig. 2A). An opposite trend was observed in the gills where both AMPA and salinity decrease caused a reduction of AChE activity (Fig. 2B). In digestive glands, contrasting patterns of AChE activity were observed: AMPA exposure significantly affected AChE activity, which was inhibited and enhanced at control (35) and lowest salinity (15) respectively (Fig. 2C); without AMPA, organisms exhibited a decrease of enzymatic activity at the lowest salinity (Fig. 2C).

Carboxylesterases (CEs) were assayed in both digestive glands and gills (Fig. 2D–I, Tab. SM2) toward three substrates, 4 nitrophenyl acetate (4NPA), 4 nitrophenyl butyrate (4NPB), and 1 naphthyl acetate (1NA). CEs activity in the digestive glands showed a consistent response across all substrates, with a significant inhibition in organisms exposed to AMPA at salinity 35: this inhibition disappeared at decreasing salinity, and significantly higher CEs activity was observed in AMPA-exposed mussels at both 25 and 15 compared to those at 35 (Fig. 2D–F). In the gills, no variations were observed across the different salinity scenarios in non-exposed mussels, while CEs activity was significantly induced in AMPA-exposed organisms at the lowest salinity (Fig. 2G–I).

3.3. *Immunocytes composition and functionality*

A significant decrease in the granulocyte to hyalinocyte ratio (GH) was observed at lower salinity values, further exacerbated by AMPA exposure, with more marked differences among all experimental groups (Fig. 3A, Tab. SM2). Phagocytosis was significantly reduced in organisms exposed at the lowest salinity level, irrespective of AMPA presence, while this chemical also had a significant effect at 25 (Fig. 3B–Tab. SM2). Lysosomal membrane stability (LMS) showed a significant reduction at decreasing salinities, with differences between AMPA-exposed and non-exposed organisms observed only at 15 (Fig. 3C–Tab. SM2). AMPA statistically increased micronuclei frequency (MN) at 35, while at lower salinities a similar effect was more evident in non-exposed mussels (Fig. 3D–Tab. SM2).

3.4. *Antioxidant defences*

In digestive glands, antioxidant enzymes were generally modulated by salinity more than AMPA (Tab. SM2). Catalase (CAT) was significantly higher in organisms exposed at 25 and 15, and interactive effects of AMPA and salinity were observed at the lowest salinity (Fig. 4A). Similarly, glutathione peroxidases (GPx) increased at decreasing salinity, despite such differences appeared as statistically significant only in AMPA-exposed organisms (Fig. 4B). Glutathione S-transferases (GSTs) showed a significantly higher activity at 25 in AMPA-treated mussels (Fig. 4C). Glutathione reductase (GR), GSH/GSSG ratio and superoxide dismutase (SOD) exhibited limited variations with significant increase only in non-exposed organisms at 25 (Fig. 4D–F). In gills, only limited responses were observed, mainly caused by salinity variation. GPx and, to a lesser extent, SOD significantly increased, while the

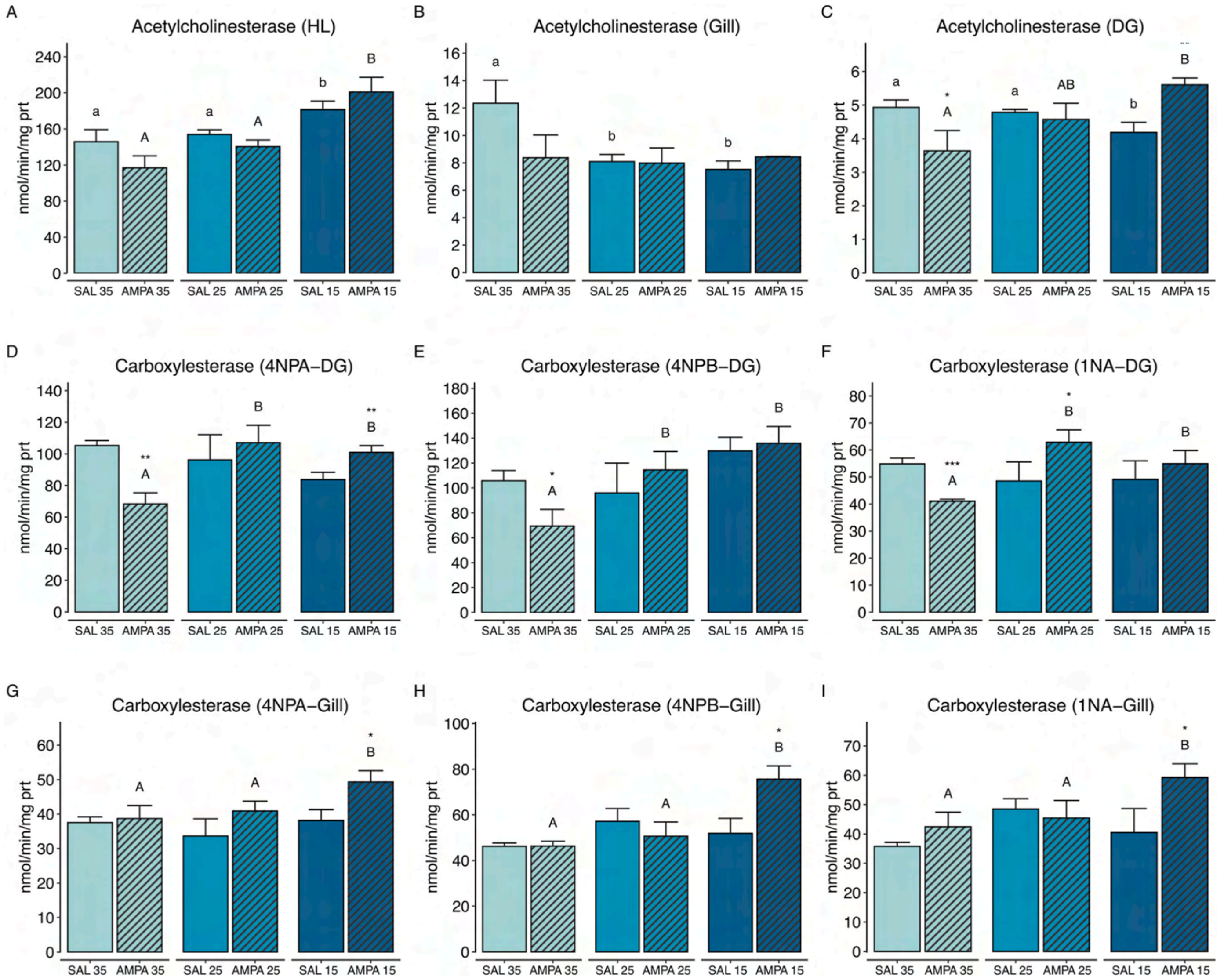


Fig. 2. Acetylcholinesterase and Carboxylesterase in *M. galloprovincialis* exposed to AMPA under different salinity scenarios. Acetylcholinesterase activity in haemolymph (A), gills (B) and digestive gland (C); carboxylesterase activity in digestive gland and gills towards different substrates: 4NPA (D, G), 4NPB (E, H), 1-NA (F, I). Results are given as mean ± standard deviation, n = 3. Lowercase letters highlight significant differences among salinity levels in treatments without AMPA (SAL 35, SAL 25, SAL 15), and uppercase letters in treatments with 0.5 μg/L AMPA (AMPA 35, AMPA 25, AMPA 15); the absence of letters indicates no significant differences among treatments. Asterisks (*) highlight significant differences among AMPA scenarios (no AMPA vs. 0.5 μg/L AMPA) at each salinity level. (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$).

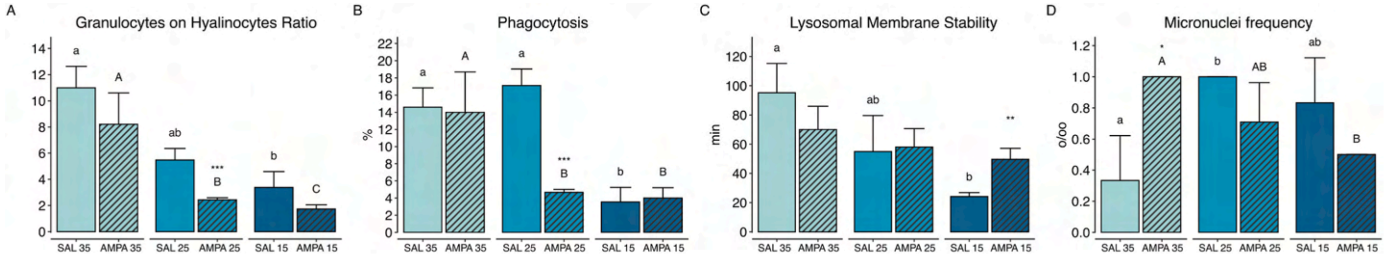


Fig. 3. Immunocytes composition and functionality in *M. galloprovincialis* exposed to AMPA under different salinity scenarios. Granulocytes to hyalinocytes ratio (A), phagocytosis rate (B), haemocytes lysosomal membrane stability (C) and micronuclei frequency (D). Results are given as mean ± standard deviation, n = 3. Lowercase letters highlight significant differences among salinity levels in treatments without AMPA (SAL 35, SAL 25, SAL 15), and uppercase letters in treatments with 0.5 μg/L AMPA (AMPA 35, AMPA 25, AMPA 15); the absence of letters indicates no significant differences among treatments. Asterisks (*) highlight significant differences among AMPA scenarios (no AMPA vs. 0.5 μg/L AMPA) at each salinity level. (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$).

GSH/GSSG ratio decreased at lower salinities (Fig. 4H–M, N).

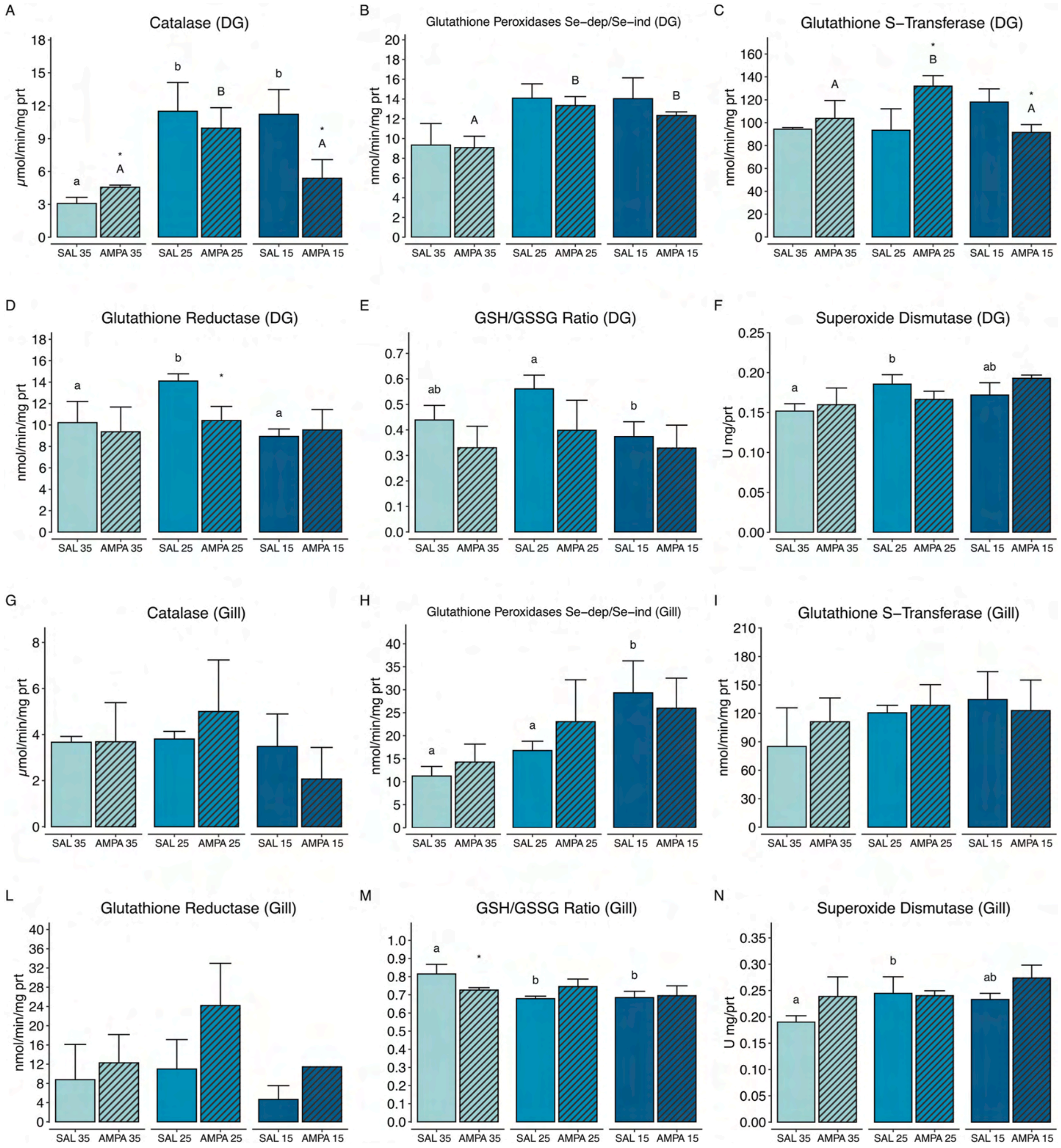


Fig. 4. Antioxidant defences in *M. galloprovincialis* exposed to AMPA under different salinity scenarios. Catalase activity (A-G), total glutathione peroxidases activity (B-H), glutathione S-transferases activity (C-I), glutathione reductase activity (D-L), GSH/GSSG ratio (E-M), superoxide dismutase activity (F-N) in digestive gland and gills. Results are given as mean ± standard deviation, n = 3. Lowercase letters highlight significant differences among salinity levels in treatments without AMPA (SAL 35, SAL 25, SAL 15), and uppercase letters in treatments with 0.5 µg/L AMPA (AMPA 35, AMPA 25, AMPA 15); the absence of letters indicates no significant differences among treatments. Asterisks (*) highlight significant differences among AMPA scenarios (no AMPA vs. 0.5 µg/L AMPA) at each salinity level. (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$).

3.5. Total oxyradical scavenging capacity and oxidative damages

Total oxyradical scavenging capacity (TOSC) towards peroxyl (ROO•) and hydroxyl (HO•) radicals was significantly lowered in digestive glands of AMPA-exposed mussels at the lowest salinity, while no significant variations in TOSC were observed in the gills (Fig. 5A–D, Tab. SM2).

Neither lipid peroxidation (LPO) nor protein carbonylation (PC)

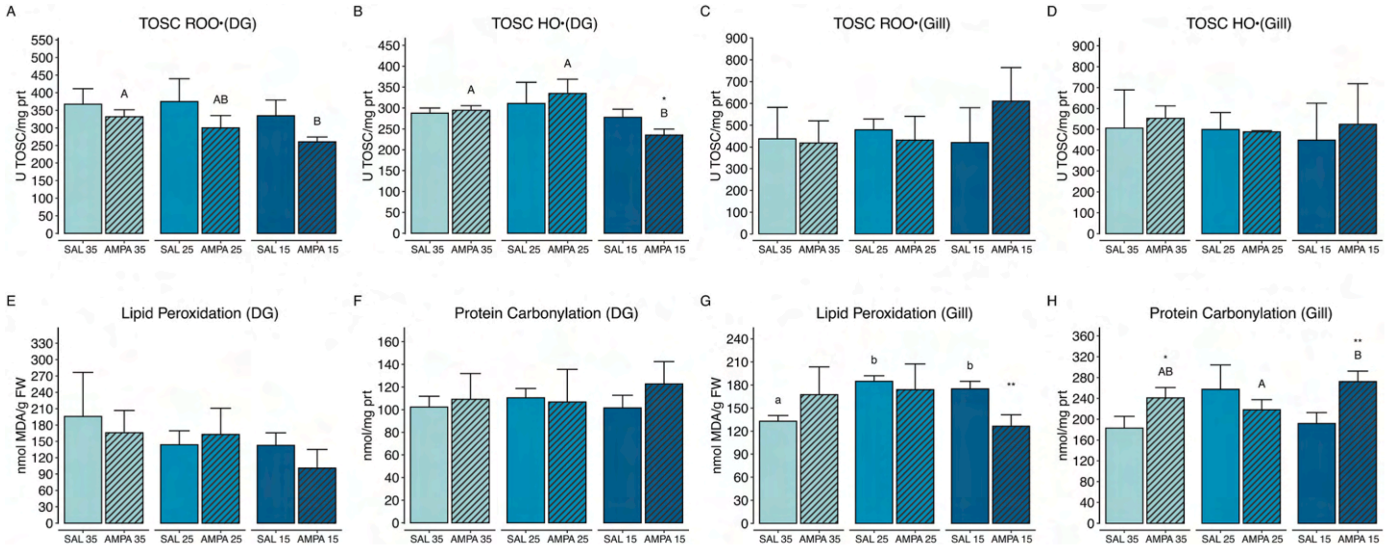


Fig. 5. Total oxyradical scavenging capacity and oxidative damages in *M. galloprovincialis* exposed to AMPA under different salinity scenarios. Total oxyradical scavenging capacity towards peroxyl radical (A-C) and hydroxyl radical (B-D), lipid peroxidation (E-G) and protein carbonylation (F-H) in digestive gland and gills. Results are given as mean ± standard deviation, n = 3. Lowercase letters highlight significant differences among salinity levels in treatments without AMPA (SAL 35, SAL 25, SAL 15), and uppercase letters in treatments with 0.5 μg/L AMPA (AMPA 35, AMPA 25, AMPA 15); the absence of letters indicates no significant differences among treatments. Asterisks (*) highlight significant differences among AMPA scenarios (no AMPA vs. 0.5 μg/L AMPA) at each salinity level. (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$).

showed statistically significant differences across salinity conditions or AMPA exposure in the digestive gland (Fig. 5E and F, Tab. SM2). Conversely, in gills, LPO levels significantly increased at lower salinities without AMPA and a significant increase of PC levels was observed at both 35 and 15 in AMPA-exposed compared to non-exposed ones (Fig. 5G and H, Tab. SM2).

3.6. *Lipid metabolism and energy homeostasis*

A marked and statistically significant increase of Acyl-CoA oxidase (ACOX) activity was observed at decreasing salinities with a similar trend in AMPA-exposed and non-exposed mussels (Fig. 6A, Tab. SM2). Concomitantly, a generalized decrease of neutral lipid (NL) content was observed, with statistically significant differences among treatments in AMPA-exposed mussels (Fig. 6B–Tab. SM2). Limited differences were observed in terms of lipofuscin (LIPO) content, which showed a significant increase caused by AMPA-exposure only in mussels at 35 (Fig. 6C–Tab. SM2).

AMPA caused a significant induction of the electron transport system (ETS) activity in the digestive gland of mussels exposed at lower salinities (Fig. 6D–Tab. SM2); also, a generalized decrease of glycogen content (GLY) was observed, especially caused by AMPA at 35, without differences between AMPA-exposed and non-exposed organisms at lower salinities (Fig. 6E–Tab. SM2).

In the gills, hyposalinity significantly increased ETS activity (Fig. 6G–Tab. SM2), with a greater magnitude of effect observed in AMPA-exposed mussels, which showed significant differences compared to non-exposed mussels at 25 and 15. Similarly, proline (PROL) content significantly increased with decreasing salinity, with this factor exerting a major effect in organisms not exposed to AMPA (Fig. 6I–Tab. SM2).

3.7. *Weight of Evidence elaboration*

The entire dataset of results was further elaborated using standardized weighted criteria based on the number, magnitude and toxicological relevance of observed variations, which allows for obtaining a synthetic hazard index and a classification to compare each experimental treatment (Fig. 7).

Compared to control mussels at 35, the WOE approach assigned a "SLIGHT" hazard to those exposed to AMPA at the same salinity and to those maintained at 25 without AMPA. The biological significance of observed effects increased the hazard classification to "MODERATE" in AMPA-treated mussels at 25 and in those exposed to the lowest salinity (15), both with or without AMPA (Fig. 7): in these 3 groups, the highest HQ value was obtained in organisms co-exposed to AMPA at 15, followed by those at 15 without AMPA, and those exposed to AMPA at 25.

The biological responses most frequently observed to have variations in the higher classes of hazard (MODERATE, MAJOR and SEVERE) were GH (in 4 treatments), ETS in gills (4), CAT in the digestive gland (4), PHAGOCYTOSIS (3), NL (3), ACOX (3), PROL (3) and GPx (3) in gills. Worthy to note, mussels at 15, with or without AMPA, had 4 biological responses in common with a "SEVERE" hazard classification (ETS, GPx and PROL in gills and ACOX), and 2 biological responses with a "MAJOR" hazard classification (GH and PHAGOCYTOSIS).

4. Discussion

Intensifying extreme flood events worldwide (Sabater et al., 2023) might increase freshwater inflows into coastal marine ecosystems, potentially resulting in transient salinity reductions at local scale, with basin-specific magnitude and spatial extent. These conditions can affect marine organisms, particularly osmoconformers, targeting key physiological processes as osmotic balance, ionic regulation, and cellular homeostasis (Matozzo and Marin, 2011). Intense and enhanced freshwater inflows may also increase the transport of land-based chemical contaminants into marine environments, as pesticides (Lefrancq et al., 2017; Topaz et al., 2018). Among these, AMPA, the main degradation product of glyphosate and recognized as toxic for aquatic biota, is increasingly detected in aquatic systems (Struger et al., 2015; Pupke et al., 2016; Wirth et al., 2021). Although the individual effects are relatively characterized, their combined impact remains largely unexplored. In this context, the present study provides new insights into the toxicological profile of AMPA under hyposaline scenarios associated with extreme flood events.

Multivariate analyses revealed that AMPA effects on the biomarker profile were significantly modulated by salinity, indicating non-additive interactions between stressors. Principal coordinates analysis (PCoA) provided a bidimensional representation showing a clear separation

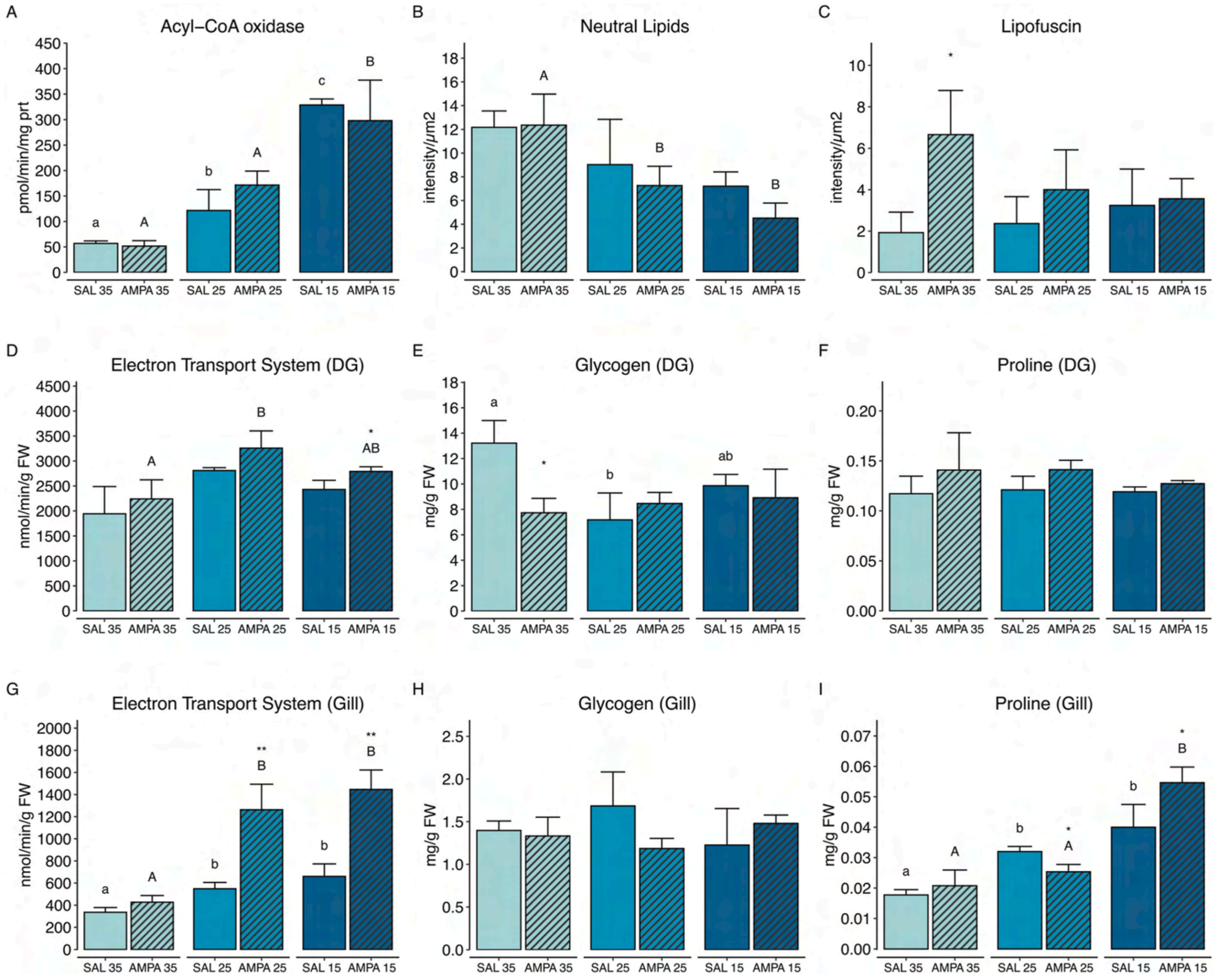
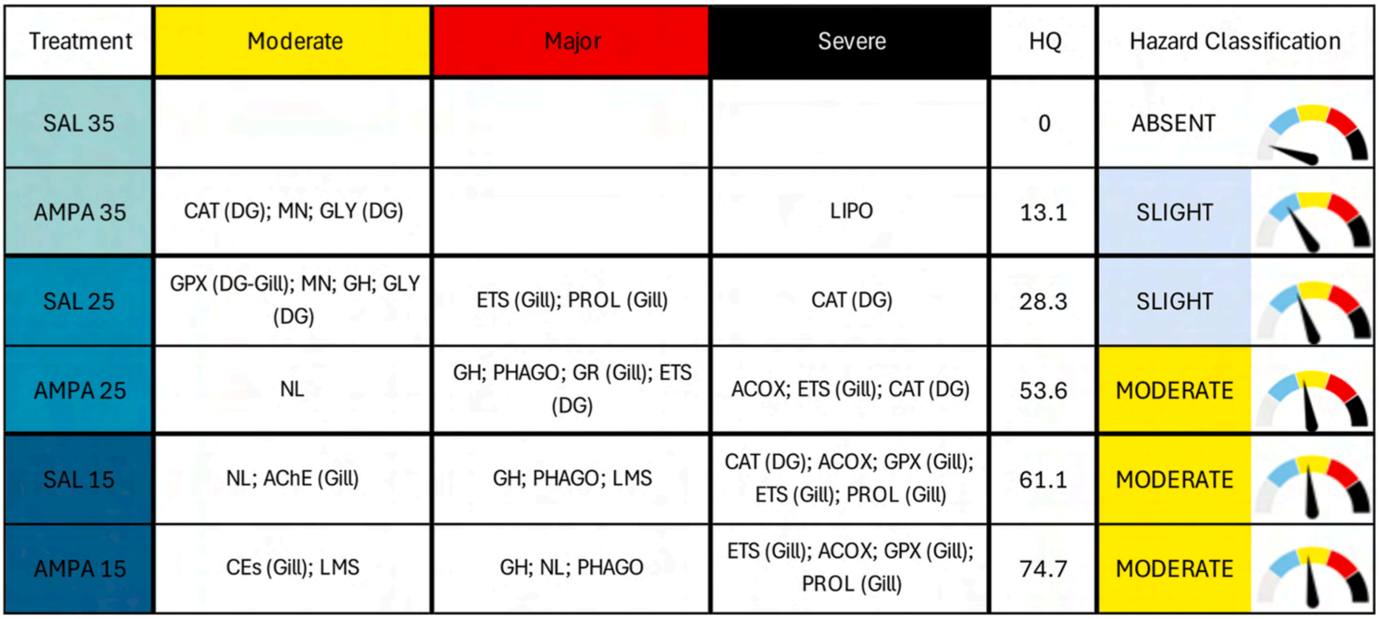


Fig. 6. Lipid metabolism and energy homeostasis in *M. galloprovincialis* exposed to AMPA under different salinity scenarios. Acyl-CoA oxidase activity in the digestive gland (A) neutral lipid content (B) and lipofuscin content (C) in 8 μm thick digestive gland sections. Electron transport system (D-G), glycogen content (E-H) and proline content (F-I) in digestive gland and gills. Results are given as mean ± standard deviation, n = 3. Lowercase letters highlight significant differences among salinity levels in treatments without AMPA (SAL 35, SAL 25, SAL 15), and uppercase letters in treatments with 0.5 μg/L AMPA (AMPA 35, AMPA 25, AMPA 15); the absence of letters indicates no significant differences among treatments. Asterisks (*) highlight significant differences among AMPA scenarios (no AMPA vs. 0.5 μg/L AMPA) at each salinity level. (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$).

between control and AMPA-exposed treatments at salinities 35 and 15, whereas partial overlap between treatments was observed at salinity 25. Notably, AMPA exposure under extreme hyposalinity resulted in the most pronounced divergence from control conditions. Biological responses associated with energy metabolism and detoxification mechanisms in the gills, lipid metabolism in the digestive gland and neuroimmune function in haemolymph strongly contributed to the separation, highlighting a salinity-dependent modulation of these processes. In parallel, biomarkers related to oxidative stress and cholinergic function both in the gills and digestive gland, with a certain contribution of energy metabolism in the digestive gland, resulted as the AMPA-mediated alterations differently modulated under tested salinity scenarios. Overall, these results suggest a context-dependent modulation of multiple biological pathways, reflected through tissue- and endpoint-specific responses shaping the observed multivariate patterns.

Within this framework, AChE activity emerged to be altered with tissue specificities. Consistent with its parent compound glyphosate and other organophosphorus contaminants, AMPA inhibited acetylcholinesterase activity under control conditions (English and Webster, 2012; Matozzo et al., 2018, 2019; Nardi et al., 2025). However, this effect was modulated by salinity, which counteracted AChE inhibition in both digestive gland and haemolymph, suggesting a response linked to osmotic stress and altered ionic balance and consistent with previously reported antagonistic interactions between hyposalinity and pesticides, likely involving neuroendocrine regulation (Velasco et al., 2018; Andrade et al., 2024). Accordingly, the use of AChE inhibition as a biomarker of pesticide exposure remains debated under environmentally realistic, multi-stressor conditions (Pfeifer et al., 2005; Tankoua et al., 2011; Lassoued et al., 2023).

Carboxylesterases also emerged as key drivers of the multivariate patterns, confirming their role in the metabolism of AMPA and glyphosate (Lajmanovich et al., 2011; Nardi et al., 2025; Tu et al., 2025). Although AMPA concentrations in tissues were not determined, CEs activity modulation suggests an interaction with detoxification pathways. The inhibition observed in the digestive gland at control salinity supports the previously hypothesized ability of AMPA to bind the



Treatment	Moderate	Major	Severe	HQ	Hazard Classification
SAL 35				0	ABSENT
AMPA 35	CAT (DG); MN; GLY (DG)		LIPO	13.1	SLIGHT
SAL 25	GPX (DG-Gill); MN; GH; GLY (DG)	ETS (Gill); PROL (Gill)	CAT (DG)	28.3	SLIGHT
AMPA 25	NL	GH; PHAGO; GR (Gill); ETS (DG)	ACOX; ETS (Gill); CAT (DG)	53.6	MODERATE
SAL 15	NL; AChE (Gill)	GH; PHAGO; LMS	CAT (DG); ACOX; GPX (Gill); ETS (Gill); PROL (Gill)	61.1	MODERATE
AMPA 15	CEs (Gill); LMS	GH; NL; PHAGO	ETS (Gill); ACOX; GPX (Gill); PROL (Gill)	74.7	MODERATE

Fig. 7. Weight of Evidence Hazard Classification. Weighted elaboration of collected results for each experimental treatment. Biological responses mostly contributing to the hazard calculation with Moderate, Major and Severe effects are given, along with the overall hazard quotient (HQ) and hazard classification.

enzyme active site (Nardi et al., 2025). Conversely, hyposalinity appeared to mask this inhibitory effect, leading to increased CEs activity, an effect previously reported in *M. galloprovincialis* under decreased salinity (Andrade et al., 2021; Bortot et al., 2025).

Immune parameters were affected by both stressors, with a clear trend toward immunosuppression under decreasing salinity and a minor role of AMPA. A progressive reduction in granulocytes to hyalinocytes ratio and in phagocytosis with decreasing salinity and AMPA exposure, suggests that both stressors can negatively affect immune efficiency, as previously observed in *M. edulis* and other marine invertebrates (Cheng et al., 2004; Gagnaire et al., 2006; Bussell et al., 2008; Kuchel et al., 2010; Nardi et al., 2025). Loss of lysosomal membrane stability, consistent with changes in the granulocytes to hyalinocytes ratio, may represent the mechanism underlying immune function impairment, as granulocytes, lysosome-rich cells, are essential for effective phagocytosis (Yang et al., 2015; Nardi et al., 2021).

Mild genotoxic effects of AMPA were observed, with increased micronuclei frequency under control salinity, consistent with previous findings in *M. galloprovincialis* and *Lymnaea stagnalis* (Schleicherová et al., 2024; Nardi et al., 2025). However, these effects were masked under hyposaline conditions, which slightly increased micronuclei formation, suggesting that osmotic stress may interfere with cell-cycle progression and DNA repair pathways (Qiu et al., 2024; Vaidya et al., 2024).

Oxidative stress is a common mechanism underlying both AMPA toxicity and salinity fluctuations, through increased ROS production and modulation of antioxidant defences (Matozzo et al., 2018, 2019; Barreto et al., 2023; Gostyukhina et al., 2023; Andreyeva et al., 2024). Overall, our results highlight a broad antioxidants activation under hyposalinity, whereas AMPA acted more selectively and interacting with salinity stress on the modulation of biotransformation mechanism.

Consistent with long-term exposure studies in *M. galloprovincialis*, antioxidant responses showed limited sensitivity to AMPA alone (Matozzo et al., 2018, 2019; Nardi et al., 2025). Combined with transcriptional evidence from short-term exposure in zebrafish embryos, this suggests that antioxidant defences may act primarily as an early compensatory response to AMPA-induced stress (Ivantsova et al., 2022). Conversely, hyposalinity caused more pronounced alterations in antioxidant enzymes, confirming its role in shaping oxidative responses (Velez et al., 2016; Moreira et al., 2016; Freitas et al., 2017). Although AMPA concentrations in organisms were not determined, GSTs activity in the digestive gland reflected the interactive effects of both stressors, suggesting this biotransformation pathway is likely involved in AMPA conjugation and elimination rather than in oxyradical metabolism, as previously reported at both transcriptional and enzymatic levels in *M. galloprovincialis* (Iori et al., 2020; Nardi et al., 2025).

As an integrated measure of antioxidant status, total oxyradical scavenging capacity (TOSC) further highlighted tissue-specific patterns of interaction between AMPA and hyposalinity. In the digestive gland, AMPA exposure under extreme hyposalinity resulted in a marked decrease in the total oxyradical scavenging capacity to counteract both peroxyl and hydroxyl radicals. Notably, this was not associated with increased protein or lipid oxidation, suggesting either the activation of compensatory mechanisms or a delayed onset of oxidative damage. In contrast, gills exhibited an opposite pattern, with clear evidence of oxidative damage despite the lack of changes in TOSC. In this context, lipid peroxidation appeared to be primarily driven by hyposalinity, confirming the effects of osmotic fluctuations on membrane lipid oxidation (Carregosa et al., 2014b; Gostyukhina et al., 2023), whereas AMPA exposure promoted protein carbonylation, as reported in mammalian cell models (Borges et al., 2023).

Organisms exposed to AMPA under hyposalinity exhibited severe alterations of peroxisomal activity, energy metabolism and osmoregulatory responses, both in digestive gland and gills. The progressive increase in ACOX activity with decreasing salinity, coupled with the depletion of neutral lipids, suggests the enhanced peroxisomal β-oxidation of fatty acids to drive lipid mobilization in order to meet increased energetic demand. This interpretation is further corroborated by the concurrent reduction in glycogen content, indicating a generalized mobilization of energy reserves under stress conditions, as previously reported in clams *R. philippinarum* exposed to hyposalinity and in fish *Leporinus obtusidens* exposed to glyphosate (Glusczak et al., 2006; Carregosa et al., 2014a). Such hypothesized increase of energy requirements associated with stress-response is also reflected by the enhanced mitochondrial activity, especially in gills of organisms under combined AMPA exposure and hyposalinity. However, increased mitochondrial respiration may also promote ROS production, linking metabolic reorganization to the observed oxidative damage.

In parallel, the salinity-dependent increase in proline levels in gills is consistent with its role in osmotic regulation under salinity stress (Li et al., 2024). Beyond its osmoprotective function, proline may also contribute to energy metabolism by supplying electrons to the mitochondrial ETS, thereby coupling osmotic adjustment with respiration and redox balance (Phang, 1985; Sharma et al., 2011). Overall, these results indicate that metabolic reorganization under combined salinity and AMPA stress involves coordinated shifts in energy allocation, osmotic regulation, and redox homeostasis.

To provide an integrative assessment of the biological alterations based on the number, magnitude, and toxicological relevance of observed responses, the dataset was analysed using a quantitative WOE approach, allowing the derivation of a synthetic hazard index for each experimental scenario. Hazard quotients increased progressively with decreasing salinity and were consistently exacerbated by AMPA exposure. Accordingly, AMPA-exposed mussels showed higher hazard levels than non-exposed organisms under the same salinity scenario, with the highest values in co-exposed organism under extreme hyposalinity, confirming a clear interaction between chemical and environmental stressors. This quantitative hazard characterization was driven by coordinated alterations across multiple biological targets and pathways. Endpoints related to immune function (phagocytosis, granulocytes to hyalinocytes ratio), antioxidant defences (CAT, GPx), and energy metabolism and osmoprotection (ETS, ACOX, proline) were consistently represented across combined stressors treatments. Additionally, these responses were consistently assigned a higher hazard classification, allowing to suggest these among the main target of tested stressors both in terms of magnitude of effect and toxicological relevance. Notably, mussels exposed to extreme hyposalinity shared several endpoints classified as “SEVERE” or “MAJOR”, indicating a consistent and systemic physiological disruption under these conditions. Overall, these findings demonstrate that salinity reduction increases the overall biological sensitivity of mussels to AMPA, promoting a multi-level stress response effectively captured by the integrative hazard index.

5. Conclusion

Overall results highlighted a major role of salinity in enhancing the hazard posed by AMPA to *M. galloprovincialis*, emphasizing the importance of considering multiple stressor interactions in the toxicological profiling of chemicals.

The integrative assessment indicates that salinity variations can exacerbate AMPA toxicity, with tissue- and endpoint-specific patterns. The increase of both climate-driven extreme events and anthropogenic pollution urgently requires the toxicological profiles of emerging contaminants to be assessed under climate change scenarios to provide a more realistic characterization. In this context, the possibility to summarize the complexity of mechanistic responses into synthetic indices through weighted criteria offers a valuable tool for risk assessment and communication, supporting science-based environmental management and protection. Considering that local conditions and ecological history can shape organism sensitivity, future studies should investigate tolerance thresholds to salinity shifts and AMPA in mussel populations across different regions.

CRediT authorship contribution statement

Veronica Vivani: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Alessandro Nardi:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Formal analysis, Data curation. **Marta Cunha:** Resources, Investigation, Formal analysis. **Giuseppe d’Errico:** Writing – original draft, Software, Formal analysis, Data curation. **Maura Benedetti:** Writing – review & editing, Supervision, Project administration. **Marica Mezzelani:** Writing – review & editing, Visualization, Conceptualization. **Francesco Regoli:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Rosa Freitas:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Stefania Gorbi:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2026.128259.

Data availability

Data will be made available on request.

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