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Unraveling the interplay between environmental microplastics and salinity stress on *Mytilus galloprovincialis* larval development: A holistic exploration

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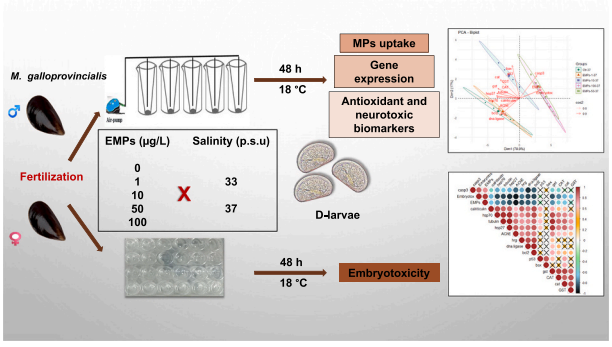
HIGHLIGHTS

- The effects of EMPs exposure and salinity are investigated for the first time in bivalves' larvae.
- Higher salinity significantly reduced the uptake of EMPs in *Mytilus galloprovincialis* larvae.
- The interactive effects increased malformations and developmental arrests of *mussel's larvae.*
- EMPs and increased salinity affected biomineralization and apoptosis processes in mussels' D-larvae.

GRAPHICAL ABSTRACT



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ABSTRACT

The rise of plastic production has triggered a surge in plastic waste, overwhelming marine ecosystems with microplastics. The effects of climate change, notably changing salinity, have shaped the dynamics of coastal lagoons. Thus, understanding the combined impact of these phenomena on marine organisms becomes increasingly crucial. To address these knowledge gaps, we investigated for the first time the interactive effects of environmental microplastics (EMPs) and increased salinity on the early development of *Mytilus galloprovincialis* larvae. Morphological assessments using the larval embryotoxicity test revealed larval anomalies and developmental arrests induced by EMPs and increased salinity. Transcriptomic analyses targeting 12 genes involved in oxidative stress, apoptosis, DNA repair, shell formation, and stress proteins were conducted on D-larvae uncovered the potential effects of EMPs on shell biomineralization, highlighting the role of Histidine Rich Glycoproteine (HRG) and tubulin as crucial adaptive mechanisms in Mytilus sp. in response to environmental shifts. Furthermore, we explored oxidative stress and neurotoxicity using biochemical assays. Our findings revealed a

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potential interaction between EMPs and increased salinity, impacting multiple physiological processes in mussel larvae. Our data contribute to understanding the cumulative effects of emerging anthropogenic pollutants and environmental stressors, emphasizing the need for a holistic approach to assessing their impact on marine ecosystems.

1. Introduction

Over the past 15 years, a substantial increase in plastic production, fueled by multiple industries and consumer needs, has led to an unprecedented flood of plastic waste flowing into the oceans (Eriksen et al., 2023; Krikech et al., 2023). The accumulated plastics in oceans can be widely classified into four levels based on their size: macroplastics (< 1 m), mesoplastics (< 2.5 cm); microplastics (<5 mm) and nanoplastics (<1 μm). Microplastics (MPs) reach the marine environment in their initial form (primary MPs) or following the degradation or fragmentation of plastic objects (secondary MPs) (Thushari and Senevirathna, 2020). The small size of MPs facilitates their absorption by marine organisms, either directly or by trophic transfer (Hu et al., 2022; Pantos, 2022). The presence of adsorbed contaminants and their role as vectors for hazardous microbes such as emerging bacterial threats and deadly viruses make MPs a threat to marine ecosystems, raising serious concerns regarding the overall health and stability of marine environments (Joo et al., 2021; Martín et al., 2022). Researchers are currently investigating the impacts of MPs on aquatic organisms (De Marco et al., 2023; Fabra et al., 2021; Missawi et al., 2020; Nanthinidevi et al., 2022), as it is crucial to understand the mechanisms impacting bioaccumulation and the ability of MPs to cause cellular and systemic effects.

Coastal environments, such as coastal lagoons, are recognized as reservoirs for MPs (Garcés-Ordóñez et al., 2022), primarily due to limited water exchange and shallow depths. This phenomenon is increasingly exacerbated by the effects of climate change, influencing the distribution and degradation of plastics within these marine environments. As a result of climate change, changes in global patterns of temperatures, precipitation, and water cycles have been reported, causing obvious changes in salinity in the world's oceans (Sathyanarayanan et al., 2021). The special report released by the Intergovernmental Panel on Climate Change (IPCC) in October 2018, warned that the extent and intensity of salinity in the coming years are likely to increase due to climate change (Masson-Delmotte et al., 2019). Salinity fluctuations induced by climate change can both perturb and increase the dispersion of MPs in these coastal ecosystems (Zhou et al., 2021). These fluctuations may result in different behaviors of MPs particles near the buoyancy threshold, subsequently altering the accessibility of MPs to marine organisms (Serra and Colomer, 2023). Increasing salinity has the potential to influence the aggregation of tiny plastic particles and trigger the salting-out phenomenon of chemicals. This, in turn, can alter the partitioning of chemicals between water and plastic materials (Liu et al., 2020; Menéndez-Pedriza and Jaumot, 2020). Furthermore, salinity affects the bioavailability and absorption of certain contaminants by marine organisms(Cui et al., 2023; Zhou et al., 2019), as well as physiological activities (feeding, respiration, and growth) and biochemical parameters (oxidative stress, immune response, and osmoregulation) (Gallo et al., 2020; Rahi et al., 2021). Although rising salinity levels may intensify the impacts of MPs pollution on the global health of marine organisms, the consequences of salinity on the accumulation and toxicity of MPs remain largely unknown.

The early life stages of bivalve mollusks represent a critical stage in their life cycle, playing a substantial role in maintaining populations and in the stability and resilience of ecosystems (Gosling, 2021). The developmental success hinges upon the quality of embryos and larvae, exerting substantial influence on the overall well-being of bivalve populations and carrying significant ramifications for the entire ecosystem (Pechenik, 2017). In marine bivalves, gamete release occurs in seawater, and, therefore, fertilization and embryonic development occur externally (Deguchi and Osada, 2020). These developmental phases showcase remarkable responsiveness to environmental stressors, exerting an impact on their vitality and intricate developmental process (Enricuso et al., 2019).

Environmental stresses generally do not occur separately (Carrier-Belleau et al., 2021) and bivalve mollusk living in coastal lagoons are easily exposed to multiple stressors including salinity variation and MPs (Du et al., 2023). Thus, these may adversely affect the normal development across all distinct life phases of these species (Bosch-Belmar et al., 2022; Sayco et al., 2019). Particularly, larval development parameters in bivalve mollusks, as broadcast-spawning species with high fecundity and a prolonged pelagic larval phase, are critical endpoints in ecotoxicological assays. Their sensitivity and ability to establish measurable dose-effect links, considering environmental stressors in both short and long terms, are crucial to assessing the overall impact of natural and anthropogenic pollutants on the reproduction, growth, and survival of these species (Jha et al., 2000; Séguin et al., 2017). In this context, the embryo-larval stages of the bivalve *Mytilus galloprovincialis* have been suggested as a global bioindicator for assessing rising salinity (His et al., 1989) and MPs pollution in coastal areas (Capolupo et al., 2018), more specifically in the Bizerte lagoon in northern Tunisia. Indeed, *M. galloprovincialis* mussels serve as excellent bio-indicators owing to their ecologically sedentary nature, large distribution, high rates of filtration and accumulation, as well as their responsiveness to various stressors (Ding et al., 2021; Freitas et al., 2017).

Previous investigations have unveiled the impact of salinity fluctuations on the development of bivalve mollusk larvae (Tezuka et al., 2013; Verween et al., 2007). Nevertheless, most previous studies have separately investigated the individual effects of MPs or salinity on the early life stages of marine bivalves (Bringer et al., 2020; Madrones-Ladja, 2002). To date, only a few studies have explored the combined effects of MPs and salinity in adult marine organisms (Capparelli et al., 2023; Dong et al., 2022; Du et al., 2023; Sui et al., 2023). But to our knowledge, no study has evaluated the interactive effects of MPs exposure and salinity variation in the early life stages of aquatic organisms. To address these knowledge gaps, this study investigates the effects of exposure, both individually and in combination, to MPs and increased salinity on mussels D-larvae. To achieve this goal, the strategy adopted is to use environmental microplastics (EMPs) that encompass a wide range of polymers, shapes, and sizes to replicate the various characteristics observed in real natural conditions.

In the current study, we examined the ingestion of EMPs by *M. galloprovincialis* mussel D-larvae, investigating whether the uptake of MPs was affected by high salinity exposure. Employing a morphological approach, we assessed the potential toxicity of MPs and increased salinity on mussels D-larvae, through their impact on larval anomalies. Additionally, a transcriptomic analysis was conducted on mussel D-larvae to explore the differential expression levels of 12 genes involved in various physiological processes such as apoptosis, DNA repair, shell formation, stress proteins, and oxidative stress aiming to examine potential interactive effects between MPs and increased salinity. Furthermore, biochemical investigations were carried out, evaluating different enzymes associated with oxidative stress (catalase and glutathione-s-transferases) and neurotoxicity (acetylcholinesterase activity) in mussel D-larvae under various EMPs and salinity treatments. Through these multi-approaches, our study aims to explore a potential interaction between EMPs and increased salinity on the early development of mussels at different levels of organization, ranging from the gene to the entire organism.

2. Material and methods

2.1. Collection and characterization of EMPs

In this study, the EMPs were collected from a sandy beach in Bizerte, located in the Northern of Tunisia (37°16′25.457" N, 9°52'38.078" E). The methodology for plastic collection and characterization is detailed in the research conducted by Zitouni et al. (2021). Following their characterization, EMPs were well mixed with an electric crusher and finely ground to obtain a homogenized mix. Subsequently, a 30 μm mesh sieve was employed to separate and retain only particles smaller than 30 μm for use in the exposure procedure.

2.2. Microplastics analysis with SEM- EDX

Scanning electron microscopy (SEM; JEOL-JSMIT100, Japan) in conjunction with an energy dispersive X-ray spectroscopy (EDX) analyzer was used to determine MPs morphology, surface elemental composition and capture images in high resolution. Representatives EMPs were randomly selected for the analysis. The particle was placed on a stab and coated with a thin layer of gold by a (JEOL JFC-1300) auto fine coater. After this preparation, an SEM coupled with an EDX was used with a voltage of 10 kV and a detector working distance of 10 mm and EDX was used to determine the elemental composition of the surface (Ben-Haddad et al., 2022).

2.3. Filtered seawater and preparation of water salinity

Filtered and ultraviolet-sterilized seawater (1 μm) was procured from the "Hergla Aquaculture Society," which specializes in the cultivation of fish larvae. In the laboratory, seawater is further filtered using a 0.2 μm pore membrane filter and stored at 4 °C until use. According to the AFNOR, 2009 (AFNOR XPT, 2009, ASSOCIATION FRANCAISE DE NORMALISATION), it is recommended to use it within two weeks, but in our case, most of the time was used within two days.

Before each experiment, the salinity of the filtered seawater was verified using a conductivity meter (ProofLineCond 3310). From this original salinity (37p.s.u), dilutions using filtered Milli-Q Water, at 0.2 μm pore membrane, were performed to obtain the final expected salinity of 33 p.s.u (corresponding to ideal salinity for embryo-larval development of mussels) (E. His et al., 1999a). A high salinity of 37p.s.u was chosen according to that recently recorded in Bizerte lagoon (Gaaloul et al., 2022; Khammassi et al., 2022).

2.4. Stock and working solutions

A stock solution of EMP (4 g/L) was prepared in filtered Milli-Q water and stored at ambient temperature. EMPs characterization in the stock solution was checked by Raman Micro Spectroscopy. Four different concentrations of EMPs (0, 1, 10, 50, and 100 μg/L), which can be found in the environment, were obtained by dilution of the EMP stock solution in FSW of corresponding salinity. Before each exposure, the working solutions were vortexed to ensure they were homogeneous.

2.5. Gametes collection, fertilization and exposure

Gamete collection, oocyte fertilization, and larvae handling were performed following the procedure described by Boukadida et al. (2016). Briefly, sexually mature farmed specimens of *M. galloprovincialis* from Bizerte were induced to spawn by thermal stimulation alternating immersion in filtered seawater (FSW) at 15 °C and 20 °C for 30 min each. For female mussels, the oocytes were emitted in the form of orange-colored ribbons, whereas the sperm of males was emitted as a white stream. Spawning females and males were isolated in 250 mL of FSW at the temperature of the bath in which the animal was laid to obtain a gametes-dense solution. Then oocytes and spermatozoa were filtered through a 100 μm and 50 μm stainless steel filter respectively to remove impurities. Gametes from different genitors were observed under an optical microscope at 10× magnification and the most reproductive pair (regular oocytes and very mobile spermatozoa) was selected for the experiment. Oocytes were fertilized by sperm suspension in a ratio of approximately 1:10, then the mixture was homogenized by a manual shaker to avoid polyspermy and to favor the meeting of gametes. Fertilization success was checked briefly under a microscope (appearance of the polar globule) and only batches of fertilized oocytes with fertilization success >90 % were used. Mussel embryos were exposed for 48 h (D-larva stage) to variable relevant environmental concentrations of EMPs and two distinct salinity levels. To study the multiple stressor effects of salinity and EMPs, ten combinations were defined: five concentration levels of EMPs (0, 1, 10, 50, and 100 μg/L) and two levels of salinity (33p.s.u as a level of normal salinity and 37p.s.u as higher salinity). FSW of suitable salinity, devoid of EMPs, was used as a negative control. Samples of D-larvae were used for chemical, morphological, and physiological analyses.

2.6. EMPs uptake in mussels' D-larvae

MPs extraction from D-Larvae was performed following the protocols of (Phuong et al., 2018; Zitouni et al., 2021). Approximately 100,000 D-larvae were subjected to a digestion process using 10 % KOH solution with incubation in an incubator at 60 °C for 24 undercontinuous agitation. Then, the digested sample goes through a sedimentation step for 4 h using potassium iodide solution (50 % KI; density: 1.55 $g.cm^{-3}$) to separate dense MPs from mineral particles. The resulting solution was filtered through three filters (0.45, 1.22, and 3 μm). To ensure the complete absence of any contamination, three blank extractions were carried out using filtered Milli-Q water (0.45, 1.22, and 3 μm). Furthermore, three other blank samples containing 10 % KOH and 50 % KI (sample-free) were also processed in parallel to account for any possible contamination. The number and types of MPs on each filter were documented using Raman Micro-Spectroscopy (RMS; Xplora®, HORIBA, Scientific, UK) coupled with microscopy and equipped with 785 nm near-infrared laser at 10- and 50-fold magnification (micrometer-scale spatial resolution <1 μm). Finally, the polymer database BioRadKnowItAll® Informatics System Raman ID Expert (2018) software was used to compare spectra corresponding to the identified MPs. No spectra were detected in the blank filters under the optimized conditions.

2.7. Embryotoxicity assay

The embryotoxicity assay used in the present study was described in detail by (AFNOR XPT, 2009; Edouard His et al., 1999b; Quiniou et al., 2005). About sixty minutes after fertilization, volumes corresponding to 250 embryos were transferred into one well of a 24-well microplate containing 2 mL of the different concentrations of EMPs solution diluted in FSW with the corresponding salinity (33 or 37 p.s.u). 24-well microplates were incubated in a temperature-controlled chamber at 18 °C for 48 h in the dark. Each condition was performed with five couples of genitors and three technical replications.

2.8. Transcriptomic

Total RNA was extracted from 40.000 larvae ($n = 5$ for each exposure condition) using acid-phenol–chloroform precipitation according to (Chomczynski and Sacchi, 1987) with TRI-Reagent (Sigma-Aldrich). Purification of the RNA was carried out by precipitation using chloroform, and the quality of RNA preparation was assessed by UV spectroscopy and agarose gel (1.5 %) in TBE electrophoresis. cDNA was synthesized from total RNA (1.5 to 2 μg) diluted in 20 μL of a reaction mixture composed of the "Random hexamer (R6)" primers, dNTP (0.5 mM), 200 U of M-MuL V H-RT (Reverse Transcriptase enzyme) and the

1× M MuLV H RT buffer as described by Dondero et al. (2005).

Relative mRNA abundances of mussel D-larvae genes encoding catalase (cat), Glutathione-s-transferases (gst), Histidine-rich glycoprotein(hrg), tubulin, Caspase-3 (casp3), tumor protein p53 (p53), dna ligase, Bcl-2-associated X protein (bax), B-cell lymphoma 2 (bcl2), heat shock protein 27 (hsp27), heat shock protein 70(hsp70) and calreticulin, were assessed in quadruplex TaqMan assays according to Negri et al. (2013). Beacon Designer v3.0 (Premier Biosoft International, Inc) was used to design probes and primer pairs (TableS1). Relative gene expression was geometrically normalized to 18S rRNA (L33452), β actin (AJ625116), and ribosomal proteinriboL27 (AJ625928) (Negri et al., 2013). The qRT-PCR was performed as described by Banni et al. (2011). A random reallocation test was used on the group mean values for the statistical analyses (Pfaffl et al., 2002).

2.9. Biomarkers of oxidative stress and neurotoxicity

Various biomarkers were assessed to analyze the toxic impacts of simultaneous exposure to microplastics and increased salinity on the D-larvae of mussels. As markers of oxidative stress catalase (CAT) and glutathione S-transferase (GST) were chosen while acetylcholinesterase (AChE) was investigated related to a neurotoxicity biomarker. The larvae were homogenized using an UltraTurrax® tissue homogenizer equipped with a Teflon pestle in a phosphate buffer (0.1 M; pH 7.5) at a ratio of 1:3 v:v while maintaining a temperature of 4 °C and operating at 800 rpm. After homogenization, the resulting mixtures were centrifuged at 10,000*g* for 20 min at 4 °C. Aliquots of the resulting supernatant (S9 fraction) were stored at −80 °C until analysis for total protein and enzyme assays. Total protein content in the homogenate was determined according to (Bradford, 1976) colorimetric assay using Coomassie Brilliant Blue G-250 as a standard. The enzymatic activity of catalase was assayed as described in (Claiborne, 1985). The variation of absorbance at 240 nm, caused by the dismutation of H2O2, was measured as a function of time consumption using a spectrophotometer ($\varepsilon = 0.04$ mM $-$ 1 cm $-$ 1). GST activity was measured by spectrophotometry at 340 nm, according to (Habig et al., 1974) ($\varepsilon = 9.6$ mM $-$ 1 cm $-$ 1). AChE activity was performed using the colorimetric method of (Ellman et al., 1961) by measuring the change in absorbance at 412 nm at an interval of 51 s for 5 min using a spectrophotometer ($\varepsilon = 13.6$ mM^{-1} cm $-$ 1).

2.10. Statistics

All data were processed statistically using the R software (https://cran.r-project.org/)and graphs on Origin Pro. All data is expressed as mean ± standard error (SE). The results were first tested for normality (Shapiro-Wilk residue test with 1 % risk) and homogeneity of variances (Levene test, 5 % risk). If conditions of use were respected, significant differences between treatments were tested with a one-way ANOVA followed by the Tukey post hoc test ($p < 0.05$). Otherwise, a nonparametric Kruskal-Wallis test was used ($p < 0.05$). The differences were considered significant when $p < 0.05$. Additionally,principal component analysis (PCA) (using Factoextra R Package) and the correlation matrix (using Corrplot R Package) were assessed to evaluate the entire data set.

3. Results

3.1. EMPs characterization in the stock solution

Analysis of the EMPs stock solution by RMS revealed the presence of 5329.65 ± 146 plastic microparticles per 100 μg. Size >3 μm accounted for 3.73 % of particles detected, while 32.52 % of particles had a size between 3 and 1.22 μm and 63.73 % had a size between 1.22 and 4.5 μm (Fig. S1). The detected EMPs with a size >3 μm consisted of 33.41 % polyethylene (PE), 30.06 % polyethylene terephthalate (PET), 20.19 % polypropylene (PP), 6.84 % polyethylene vinyl acetate (PEVA), 5.2 % high-density polyethylene (HDPE) and 4.27 % low-density polyethylene (LDPE). Furthermore, the detected EMPs with a size between 3 and 1.22 μm consisted of 23.23 % PE, 25.75 % PET, 19.16 % PP, 9.49 % PEVA, 14.14 % HDPE and 8.2 % LDPE. Eventually, EMPs whose size was between 1.22 and 0.45 μm were also identified as 35.39 % PE, 29.47 % PET, 13.8 % PP, 8.9 % PEVA, 7.45 % HDPE and 4.9 % LDPE (Fig. S2).

3.2. Microplastic characteristics

Analysis with SEM-EDX was used to analyze the elemental composition of randomly selected EMPs particles and results showed different alterations and specific materials appearing on the surface of the fragment particle. The surface is convex, showing varying degrees of damage including irregularities, fractures, and degradation, and a scaly appearance (Fig. S3a). The fragment analyzed by EDX revealed a strong carbon peak (77.97 %) and an oxygen peak (20.20 %). Another peak of Silicon is observed (Si, 1.09 %) followed by calcium (Ca, 0.74 %), (Fig. S3b).

3.3. Loads and types of EMPs ingested by mussel D-larvae

The quantification and identification of particles per 100,000 exposed D-larvae were conducted using Raman microspectroscopy analysis. The concentration of EMPs in the control group was 8.63 ± 1.29 particles/10^5 D-larvae under the control salinity condition (33 p.s.u), and 8.93 ± 0.74 particles/10^5 D-larvae under the elevated salinity condition (37 p.s.u). Noticeably, with the rise in exposure concentration, there was a significant increase in the number of particle/10^5 D-larvae: EMPs-1 (88.47 ± 8.2 particles/10^5 D-larvae), EMPs-10 (151.85 ± 15.43 particles/10^5 D-larvae), EMPs-50 (251.82 ± 19.37 particles/10^5D-larvae), and EMPs-100 (328.89 ± 25.38 particles/10^5 D-larvae) (Fig. 1a). Interestingly, high salinity of 37 p.s.u reduce significantly EMPs uptake by mussels' D-larvae: EMPs-1 (53.03 ± 5.18 particles/10^5 D-larvae), EMPs-10 (66.05 ± 7.39 particles/10^5 D-larvae), EMPs-50 (89.34 ± 7.96 particles/10^5 D-larvae), and EMPs-100 (121.46 ± 11.08 particles/10^5 D-larvae) (Fig. 1b). In the control group for both salinities, no particles from the >3 μm and (3–1.22) μm size ranges were detected, while particles <1.22 μm were identified. Contrastingly, within the other exposed groups at both salinity levels, particles from all size ranges (>3 μm, (3–1.22) μm, <1.22 μm) were detected, with the most significant presence being observed in the <1.22 μm size range (Fig. 1c and d). Notably, PE emerged as the primary polymer type, followed by PET in all exposed groups. Exposure of D mussel larvae to an increased salinity didn't affect the predominance of polymer types (Fig. 2a and b).

3.4. Effect of EMPs and salinity on mussels' embryo-larval development

The developmental anomalies of mussels D-larvae in response to EMPs were assessed (Fig. 3a), revealing a dose-response effect. Larval abnormality rate was observed in all the conditions being the most pronounced at the two highest concentrations of 50 and 100 μg/L at the control salinity condition (33p.s.u), with respectively 37.66 ± 2.51 % and 43.66 ± 2.08 %. Elevated salinity significantly increased the larval abnormality rate from 9 ± 1.0 % in salinity 33p.s.u to 66.33 ± 1.52 % in salinity 37p.s.u. The increase in salinity significantly amplified the toxicity of EMPs, resulting in a considerable rise in larval abnormality rates from the lowest EMPs concentration of 1 μg/L, highlighting a significant increase to 70.66 ± 1.15 % at 37 p.s.u in contrast to 14.33 ± 2.0 % at 33 p.s.u. Our findings revealed that developmental arrest was the most prevalent type of larval abnormality observed consistently across all EMPs concentrations and salinity levels. A biphasic response to EMPs exposure was noted: lower concentrations (1 and 10 μg/L) primarily induced mantle malformations, while higher concentrations (50 and 100 μg/L) predominantly led to shell malformations (Fig. 3b and c). High salinity significantly increases shell anomalies relative to mantle anomalies for all EMPs concentrations.

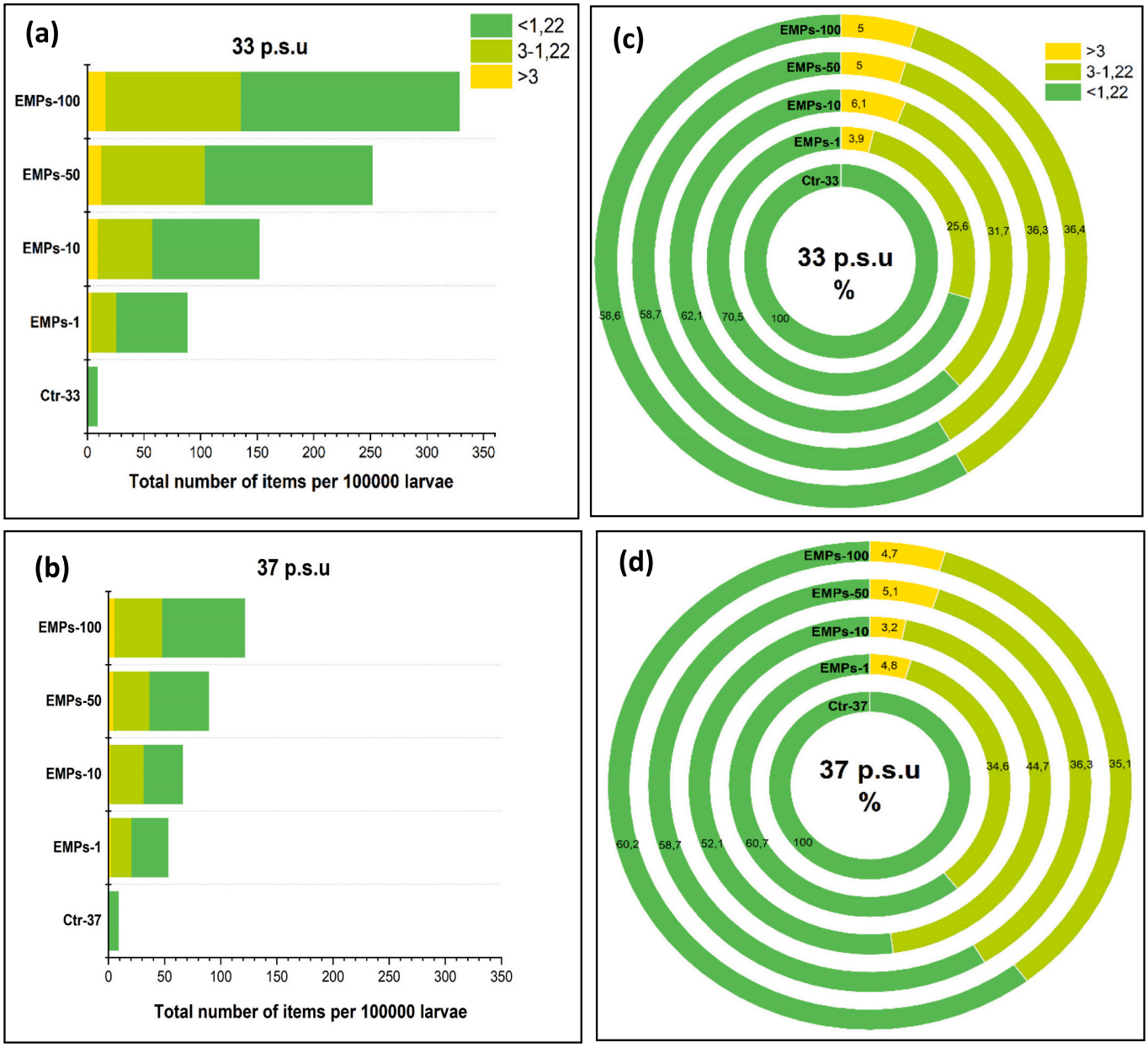

Fig. 1. Analysis of EMPs identified using Raman microspectroscopy in mussels' D-larvae exposed to different concentrations of EMPs and salinity levels (33 p.s.u and 37 p.s.u), isolated and categorized across size ranges >3 μm, (3–1.22) μm, and <1.22 μm. The findings are represented in stacked bar charts (a,b), illustrating the total amount of items observed in 100,000 D-larvae, and in doughnut charts (c,d), showcasing the percentages of EMPs present in 100,000 mussels' D-larvae.

3.5. Gene expression regulation under exposure to EMPs and salinity

The mRNA expression levels of genes involved in antioxidant defense, namely cat and gst, in apoptosis, namely Caspase-3 and Bax, in DNA repair, namely P53, DNA-ligase and Bcl-2, in shell formation, namely hrg and tubulin and in response stress, namely hsp27, hsp70, and calreticulin, were expressed in relation to the average expression of three reference genes (18S rRNA, β-actin and riboL27) to understand the effect of EMPs and salinity, alone or combined, on mussels' D-larvae (Fig. 4). Exposure to an increasing range of EMPs at control salinity condition (33 p.s.u) significantly induces gene expression levels of cat, hrg, tubulin, p53,dna ligase, bcl2,Remarkably, following a 48-h exposure to heightened salinity of 37 p.s.u compared to 33 p.s.u, mussels' D-larvae exhibited a notable upregulation in gene expression levels of cat (2.52 ± 0.28), gst (3.16 ± 0.35), tubulin (3.87 ± 0.43), p53 (2.47 ± 0.27), dna ligase (2.87 ± 0.32), bax (2.98 ± 0.33), hsp27 (4.15 ± 0.46), and calreticulin (2.85 ± 0.31). The combined exposure to high salinity and EMPs results in a notable increase in the expression level of gst at the lowest EMPs concentrations EMPs-1 and EMPs-10, with values of 2.43 ± 0.35 and 3.31 ± 0.32, respectively. We've observed an increase in the expression levels of apoptosis-related genes casp3 and bax consistently across almost all exposure concentrations. Nevertheless, there was a significant decrease in the expression levels of the DNA-ligase and hsp70 genes observed at the highest concentrations of EMPs-50 and EMPs-100. Additionally, noteworthy repression was noticed specifically for the hrg gene under the EMPs-100 condition, with a value of 0.7 ± 0.08.

3.6. Oxidative stress and neurotoxicity modulation in response to EMPs and salinity

We conducted assays on two oxidative stress biomarkers, catalase (CAT) and glutathione S-transferase (GST), as well as one neurotoxicity

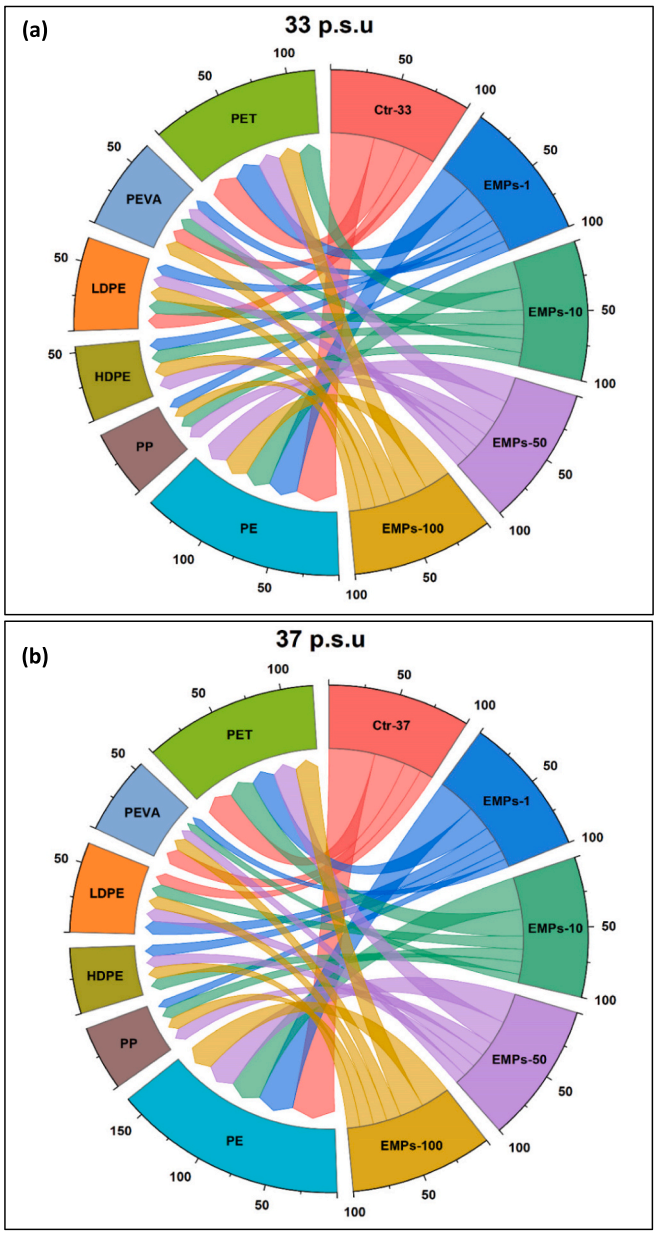

Fig. 2. Chord diagrams illustrating the composition of polymer-based EMPs identified via Raman microspectroscopyin mussels' D-larvae exposed to different concentrations of EMPs under (a) salinity control condition and (b) elevated salinity. Control 33 p.s.u (Ctr- 33), Control 37 p.s.u (Ctr- 37), Environmental Microplastics 1 μg/L (EMPs- 1), Environmental Microplastics 10 μg/L(EMPs- 10), Environmental Microplastics 50 μg/L(EMPs- 50), Environmental Microplastics 100 μg/L(EMPs- 100), Polyethylene (PE), Polypropylene (PP), High-density polyethylene (HDPE), Low-density polyethylene (LDPE),Polyethylene vinyl acetate (PEVA) andPolyethylene terephthalate(PET).

biomarker, acetylcholinesterase (AChE), in the D-larvae of mussels exposed to varying combinations of EMPs and salinity levels (Fig. 5).

At a physiological salinity of 33p.s.u., larvae demonstrated a significant increase ($p < 0.05$) in CAT activity when exposed to EMPs from a concentration of 10 μg/L, while GST activity showed a significant increase ($p < 0.05$) only at the concentration of 100 μg/L. Regarding AChE activity in larvae exposed to EMPs at a salinity of 33p.s.u., significant inhibition ($p < 0.05$) was observed at the higher EMP concentrations of 50 and 100 μg/L.

Furthermore, larvae subjected to an elevated salinity of 37p.s.u. displayed a significant increase ($p < 0.05$) in all assessed biomarkers compared to those at 33p.s.u. Exposure to both EMPs and the heightened salinity of 37p.s.u. they have initially amplified CAT and GST activities, reaching their peak increase at 10 μg/L, followed by a significant decline ($p < 0.05$) at 50 and 100 μg/L. Importantly, this exposure resulted in a significant decrease ($p < 0.05$) in AChE activity with increasing concentrations.

3.7. Principal component analysis (PCA) and correlation matrix

The results obtained from the PCA analysis (Fig. 6a), which included multiple parameters such as embryotoxicity data, CAT, GST, AChE activities, EMPs loads, and gene expression following exposure to various EMPs concentrations at physiological salinity of 33p.s.u, revealed that the first axis (71.6 %) was mainly influenced by GST, CAT, embryotoxicity, and EMPs loads. Meanwhile, the gene expression of hrg, calreticulin, tubulin, hsp27, and p53 contributed to the second axis (14.7 %). Based on these results, it was observed that only the control group was distinct from the other groups. Nevertheless, when analyzing the data collected from simultaneous exposure to EMPs and high salinity, the PCA (Fig. 6b) indicated overlapping within the control and EMPs-1-37 groups, as well as between EMPs-50-37 and EMPs-100-37. Notably, only the EMPs-10-37 group exhibited distinct separation from the rest. Specifically, the primary axis (70.8 %) in the PCA analysis was predominantly affected by the gene expression of bax, p53, and casp3 (apoptotic genes). Conversely, the secondary axis (17 %) was constituted by embryotoxicity, EMPs loads, as well as the gene expression of stress proteins hsp70 and hsp27.

Ultimately, the Pearson correlation matrix (Fig. 7a) underscored strong positive correlations among mussels' D-larvae exposed to EMPs, particularly evident between embryotoxicity and EMPs loads, CAT, cat, and hrg gene expression. Furthermore, the correlation matrix indicated a negative correlation between AChE and nearly all other parameters. Additionally, in mussels' D-larvae exposed to high salinity and EMPs (Fig. 7b), a strong negative correlation was observed between embryotoxicity and AChE, CAT, stress proteins (hsp27 and hsp70), shell formation proteins (tubulin and hrg), as well as DNA repair genes (dna ligase bcl2).

4. Discussion

Skyrocketing plastic production has caused an increase in plastic waste, overwhelming marine ecosystems with MPs. Simultaneously, the impact of climate change, notably increased salinity, has influenced the distribution and behavior of these MPs, altering their buoyancy, sedimentation, and dispersion within the coastal marine environment. Moreover, salinity can modify the capacity of MPs to absorb harmful substances due to alterations in the water's physicochemical properties (Liu et al., 2020; Menéndez-Pedriza and Jaumot, 2020). Nevertheless, despite the increasing acknowledgment of this relationship, investigations into the toxicity of MPs concerning variations in salinity among marine organisms remain constrained. Understanding the implications of these interactions, especially during early developmental stages, remains an important area of research to assess ecological impacts. Guaranteeing the well-being and survival of these vulnerable early life stages is of crucial importance for the conservation of biodiversity, the reinforcement of marine food chains, and the upkeep of the general ecological equilibrium within coastal and marine ecosystems (Gosling, 2021; Pechenik, 2017). This groundbreaking study aimed, for the first time, to investigate the potential interaction between increased salinity and artificially degraded EMPs collected from the beach of the Bizerte lagoon in northern Tunisia, during the early life stages of Mediterranean mussel *M. galloprovincialis*.

The SEM/EDX technique employed in this study has been notably advantageous, providing detailed surface structure characteristics of the EMPs' particles as well as distinctive elemental composition signatures. The micrograph illustrates varying degrees of damage evident on the

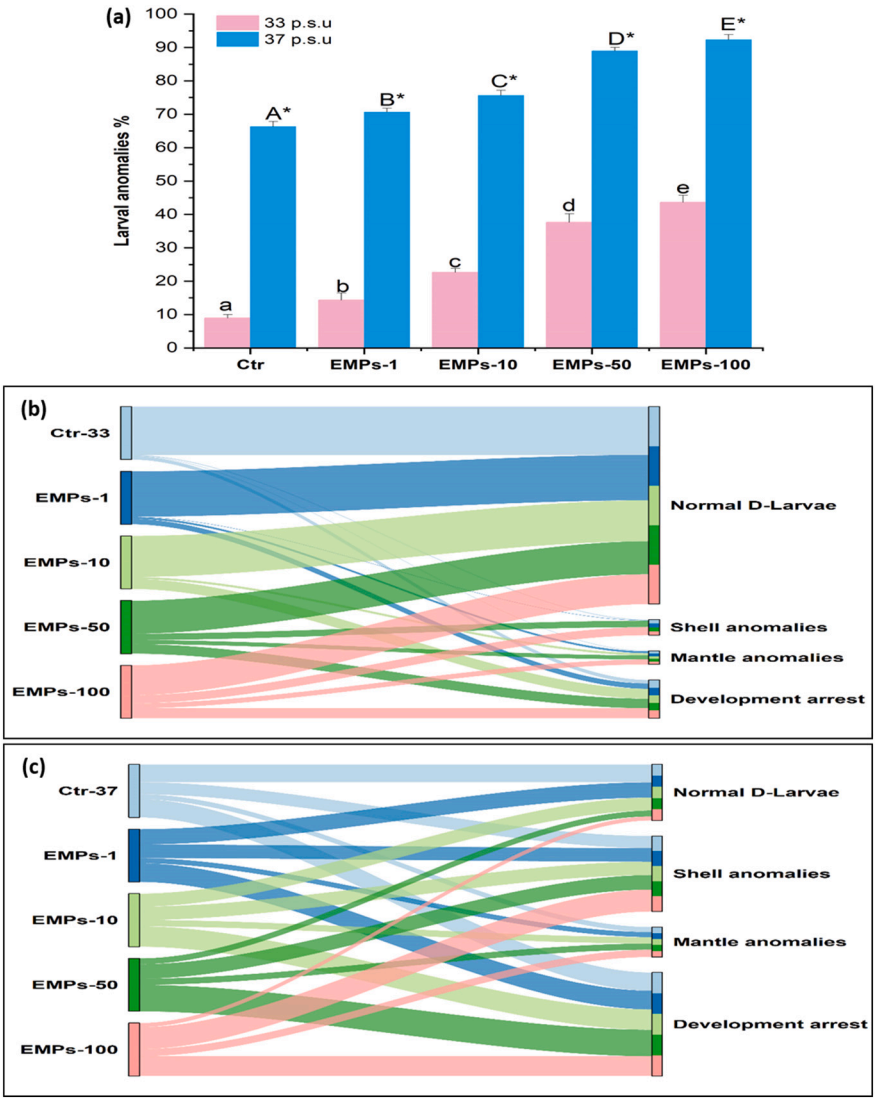

Fig. 3. (a) Total larval anomalies (Means ± SD) caused by EMPs and salinity variation. Different letters indicate significant differences between treatments, (*) presents a significant difference compared to salinity 33 p.s.u ($N = 5$, $p < 0.05$, Kruskal-Wallis). The different types of observed anomalies are elaborated upon in a Sankey diagram depicting various malformations observed in the D larvae of mussels exposed to different EMP concentrations, highlighting differences under (b) standard and (c) high salinity conditions.

irregular surface, potentially arising from the mechanical and oxidative aging process. The irregular shapes in microplastics might stem from the fragmentation of larger plastic materials by wind or storm waves. Furthermore, abrasion and degradation of EMPs' surfaces may result from exposure to high temperatures, potentially exacerbated by seawater salinity, and prolonged exposure to ultraviolet radiation from the sun in the sampling area, leading to photo-oxidation (Wang et al., 2017). The modifications in the surface texture of EMPs could result in an increase in surface area, which represents a potential mechanism for enriching surface concentrations of other highly toxic sorbed pollutants (Yuan and Xu, 2023; Zhong et al., 2023). The EMPs used in this study and analyzed by EDX showed strong peaks of C and O and lesser peaks of Si and Ca. The basic monomers are primarily composed of carbon, hydrogen, and oxygen, which combine to form the constituent polymers of plastics (Aidene et al., 2020). Although these elements may originate from the presence of organic particles (diatomite, which contains Ca and Si), these materials are commonly derived from petroleum and natural gas. Calcium carbonate and silicon dioxide, two inorganic elements incorporated as additives to base plastics, are widely used to improve specific mechanical properties or to reduce production costs.

Following exposure to varying concentrations of EMPs, either with or without a concurrent increase in salinity, an investigation was conducted to examine the presence, quantity, and type of MPs in mussel' D-larvae. Unexpectedly, a limited number of EMPs particles <1.22 μm were detected in the control group. This could be due to possible contamination occurring during the exposure experiment. The data obtained in this study showed that MPs were easily ingested by mussel larvae, with absorption rates increasing with increasing MPs concentration. The study by Sprung (1984) found that when mussel larvae were exposed to an increasing range of the alga *Isochrysis galbana* (0 to 10^4 cells mL^{-1}), they adapted their feeding rate according to the availability of external food sources. These results suggest that similar feedback regulation could occur in response to increasing concentrations of MPs. In this study, the majority of particles detected in mussel D-larvae were <1.22 μm, fewer particles between 1.22 and 3 μm and a few were > 3 μm. A growing understanding of plastic pollution in marine environments highlights the likelihood of nuanced effects caused by micro-to nano-sized particles, attributed to their size alignment with particles preferred for consumption by marine zooplankton (Galloway and Lewis, 2016). Recent studies have shown ingestion of 3 μm polystyrene-MPs by Mediterranean mussel D-larvae with increased MP uptake throughout developmental progression and at increasing MPs concentrations

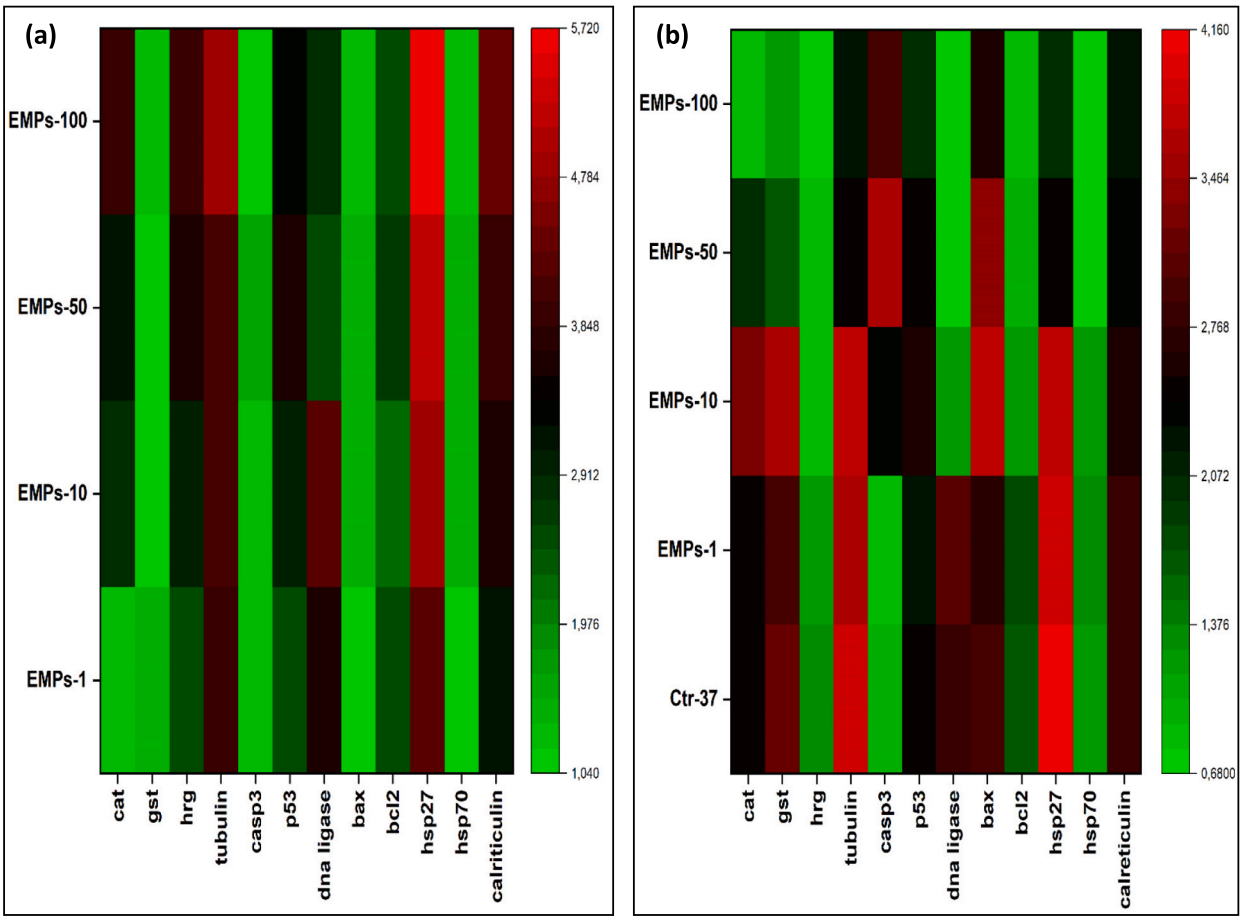

Fig. 4. Heat map visualization of RT-qPCR analysis of mRNA levels in mussel D larvae exposed to different concentrations of EMPs and salinity levels, highlighting differences between treatments based on fold change values under (a) control salinity condition and (b) elevated salinity. Relative quantification of each gene expression level was normalized according to the 18S rRNA, β actin, and ribosomal protein riboL27 gene expression. Color intensity represents the mRNA expression values relative to the Ctr-33 (control 33 p.s.u). Red represents up-regulation, and light green represents down-regulation. Rows: treatment; column: genes.

(Capolupo et al., 2018; Franzellitti et al., 2019). Notably, in the control group, only PE, PET, LDPE, and PEVA were detected. Moreover, PE consistently remained the predominant polymer across all exposure groups, followed by PET, HDPE, LDPE, PEVA, and finally PP. The polymer levels found in the mussel larvae reflect their abundance in their natural environment (Bizerte Lagoon). This finding aligns with prior studies that have highlighted the prevalence of PE particles in mussels (Toumi et al., 2019) and in seawater (Wakkaf et al., 2020) collected from the Bizerte region. Interestingly, we observed an interesting trend where a high salinity level of 37 p.s.u significantly reduced the uptake of EMPs by mussels' D-larvae. Research suggests that extreme salinity fluctuations can affect the filtration rate, feeding efficiency, and the capability of mussel larvae to capture and ingest particles (Pourmozaffar et al., 2020). High or low salinity conditions may alter the larvae' physiology of the brooding flat oyster *Ostrea angasi*, thereby reducing their capacity for optimal development (Cole et al., 2016).

Due to their increased vulnerability to a wide array of chemical compounds, the early developmental stages of mussels are frequently employed as pivotal models in ecotoxicological investigations and preliminary screenings, particularly within the context of climate change impacts (Boukadida et al., 2022, 2016). Additionally, in recent years, the early developmental stages of mussels have emerged as valuable models in assessing the toxicity of microplastics, with studies focusing on the effects of specific commercial plastics (Capolupo et al., 2018; Franzellitti et al., 2019). Despite these investigations, there remains a notable research gap regarding the assessment of EMPs in marine organisms, notably in the case of mussel D-larvae. In this study, various approaches were employed to assess the effect of EMPs on the development of early stages of Mediterranean mussels. Specifically, the larval embryotoxicity test, a sensitive and standardized assay, was used to investigate the impact of EMPs on larval development and the potential emergence of anomalies or developmental arrest. Surprisingly, we observed a significant increase in larval anomalies even at the lowest tested environmental concentration of 1 μg/L. Using a single commercial type of MPs, (Balbi et al., 2017) reported a significant effect of amino-modified polystyrene (PS-NH2) on *M. galloprovincialis* D-larvae at 48 h post-fertilization (hpf) beginning at 10 μg/L. They observed a remarkable rise in the percentage of anomalies at concentrations ≥2.5 mg/L, highlighting an EC50 value of 142 μg/L. These findings are consistent with our findings, revealing a clear dose-dependent relationship, whereby increasing exposure concentrations correspond to a reduction in normal larval development. Contradictory outcomes regarding the influence of exposure on the development of bivalve larvae have been noted, suggesting potential inter-species variability. Rist et al. (2019) observed an increase in malformations in *M. edulis* larvae, which exhibited a dose-dependent relationship when exposed to concentrations spanning from 0.42 to 282 μg/L of 2 μm polystyrene throughout a 15-day duration. Also, the development of oyster larvae was altered when exposed to HDPE microplastics ranging from 4 to 20 μm at concentrations between 0.1 and 10 mg/L (Bringer et al., 2020). However, Capolupo et al. (2018) found no evidence of developmental abnormalities in Mediterranean mussel larvae when exposed to concentrations of up to 10^5 particles/mL of 3 μm polystyrene beads without surfactants. This variability in toxicity on mollusks could be due to

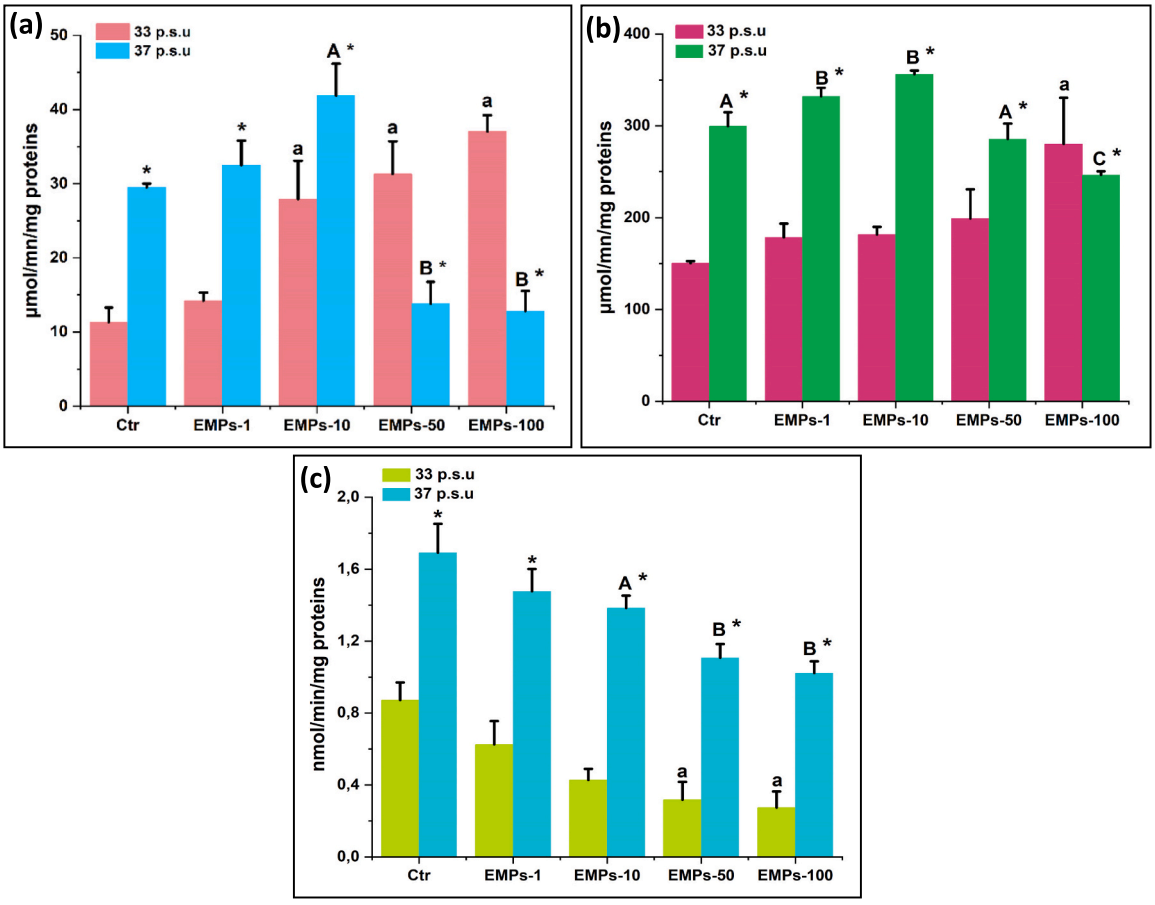

Fig. 5. Effects of EMPs and salinity on enzymatic activities in mussel D-larvae: (a) Catalase (CAT), (b) Glutathione S-transferase (GST), and (c) Acetylcholinesterase (AChE) activity. Different letters indicate significant differences between treatments, (*) presents a significant difference compared to salinity 33 p.s.u ($N = 5$, $p < 0.05$, Tukey's Post Hoc test).

variations in MPs characteristics, such as their size, as evidenced by prior studies (Ding et al., 2023; Jeong et al., 2016).

Although most bivalves are generally euryhaline and can survive in a wide range of salinity, extreme salinity levels can induce significant disparities in osmotic pressure between the environment and cellular tissues, causing stress in marine bivalves, limiting both their propagation, reproduction, and even resulting in mortality under severe conditions (Blanco et al., 2022; Ivanina et al., 2020; Pourmozaffar et al., 2020). The consequences of disturbances in osmoregulation can be significantly more pronounced during vulnerable larval stages, causing developmental irregularities (Santos et al., 2020) and increased sensitivity to other environmental stressors (Carvalho et al., 2019). Findings from this study highlight a significant association between elevated salinity levels and increased rates of abnormalities during larval stages. Regardless of the salinity level applied, we observed developmental arrest as the most prominent larval anomaly. Among other anomalies, our data indicated that an increase in salinity to 37p.s.u predominantly led to shell malformations. This is in agreement with the findings of Santos et al. (2020), who demonstrated that embryo-larval development of the yellow clam *Mesodesma matroids*, especially the length of the shells and survival rate, varied depending on salinity levels. Huo et al. (2014) showed that the survival and growth rate and shell height of the *Crassostrea hongkongensis* D-larvae were significantly lower at the high salinity (30) than at the low and medium salinities (15 and 23). Furthermore, within our dataset, we observed that increased salinity significantly heightened the toxicity of EMPs, leading to a substantial rise in larval abnormality rates. Notably, this effect was observable even at the lowest concentration of EMPs. In the literature, the impact of salinity on the accumulation and toxicity of MPs remains relatively underexplored. To date, research has addressed the combined effects of nanoparticles (NP) and salinity in bivalve mollusks (Falfushynska et al., 2023; Noor et al., 2021). However, to our knowledge, only one study has investigated the interactive effects of exposure to microplastics (MPs) and variations in salinity in adult oysters (Du et al., 2023). It was demonstrated that salinity can influence the bioavailability and uptake of certain contaminants by euryhaline invertebrates, as well as affect the physiological adjustments responsible for osmotic and ionic equilibrium (Capparelli et al., 2017).

The increase in malformations and developmental arrests of mussel larvae may be attributed to molecular-level alterations. To investigate this relationship, a real-time PCR transcriptional study was undertaken to elucidate the interplay between exposure to EMPs and fluctuations in salinity regarding the transcriptional regulation of various genes associated with specific physiological processes within the first 48 h of mussel larvae development. These processes involve apoptosis, DNA repair, shell formation, stress proteins, and oxidative stress.

The investigation focused on the expression of genes related to apoptosis and DNA repair. Particularly, the observed increase in P53 levels within mussels' D-larvae exposed to EMPs, irrespective of high salinity stress, suggests that the heightened P53 expression was most likely an immediate response induced by the microplastics in both conditions. This aligns with (Auguste et al., 2020), who reported the upregulation of P53 in mussels following exposure to 10 µg/L PS. This led us to hypothesize that P53 plays a role in defending against adducts caused by EMPs. Otherwise, exposure of mussel D-larvae to a single stress factor, either EMPs or high salinity, increased DNA ligase. These data, combined with the elevation of P53, suggest that the cell primarily activates the DNA repair mechanism by stimulating its cellular

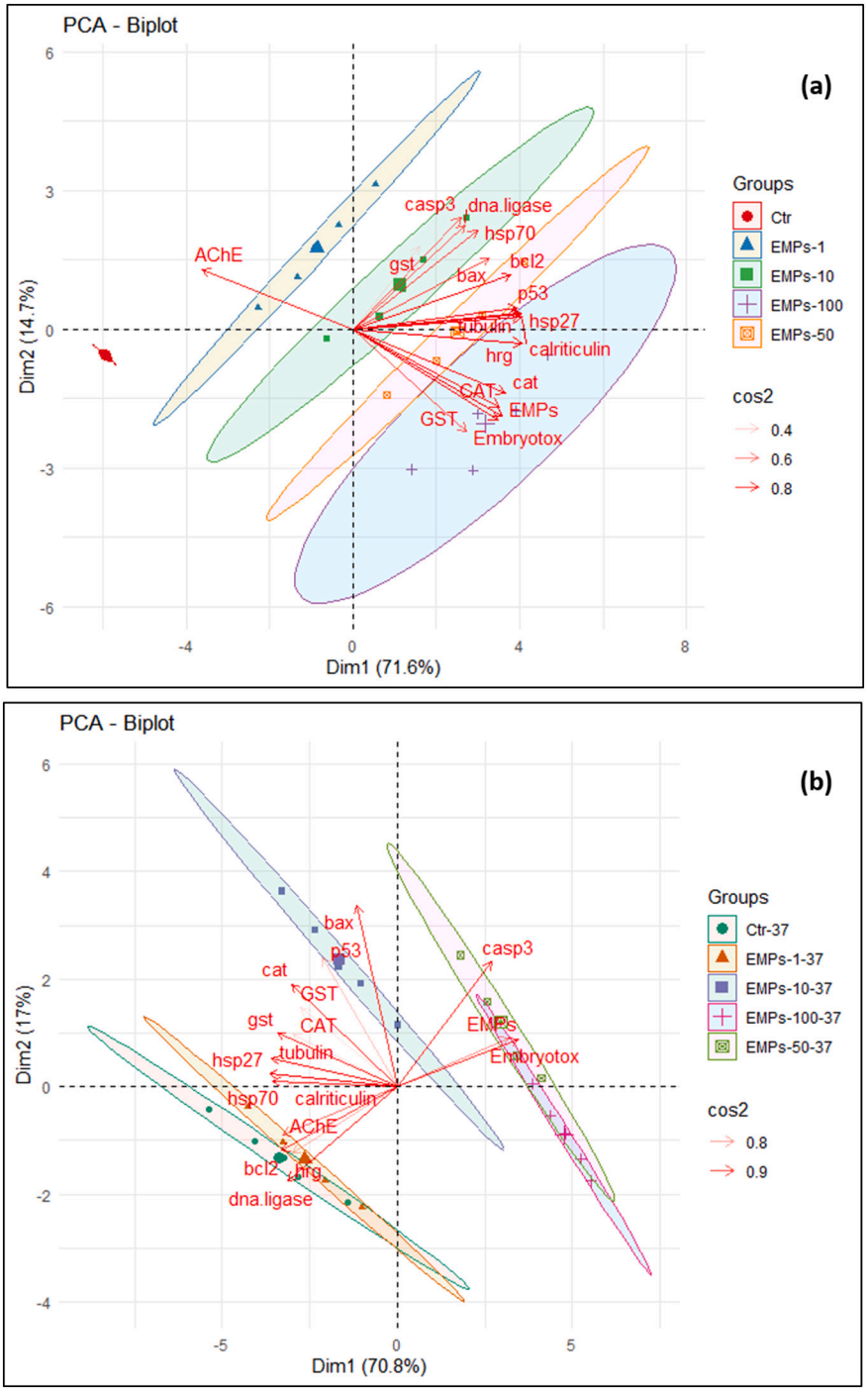

Fig. 6. PCA analysis of multiple parameters in mussel D-larvae exposed to different concentrations of EMPs and salinity levels. Embryotox (Embryotoxicity), EMPs (EMPs uptake), Catalase activity (CAT), AChE activity (AChE), GST Activity (GST), genes' expression (cat, gst,hrg, tubulin, casp3, p53, dna ligase, bax, bcl2, hsp27, hsp70, and calreticulin).

machinery by activating these genes. Furthermore, the role of the tumor suppressor gene P53 is to repair DNA following damage and protect cells from initiating the apoptosis process (Banni et al., 2009). In this study, co-exposing mussel larvae to EMPs and high salinity induced apoptosis, as indicated by upregulated expression of casp3, bax, and p53 genes, coupled with inhibition of the DNA repair system. This indicates the adaptability of mollusks' systems in response to varying salinity, contaminants, and exposure concentrations. The involvement of p53, casp3, and bax in the process of triggering apoptosis in the mussel *Mytilus galloprovincialis* exposed to 50 μg/LEMPs has already been demonstrated (Romdhani et al., 2022).

The genes involved in shell biomineralization were significantly upregulated with all applied concentrations of EMPs. A previous study demonstrated the expression of HRG and tubulin within the initial 48 h of development in various pure and hybrid species of *Mytilus* sp. under standard growth conditions, validating their significant involvement in the early phases of shell development (Mlouka et al., 2019). As a general mechanism, HRG in mussel larvae regulates shell formation by creating an acidic environment at calcification sites, crucial for calcium carbonate deposition. Its modulation of this process influences shell matrix

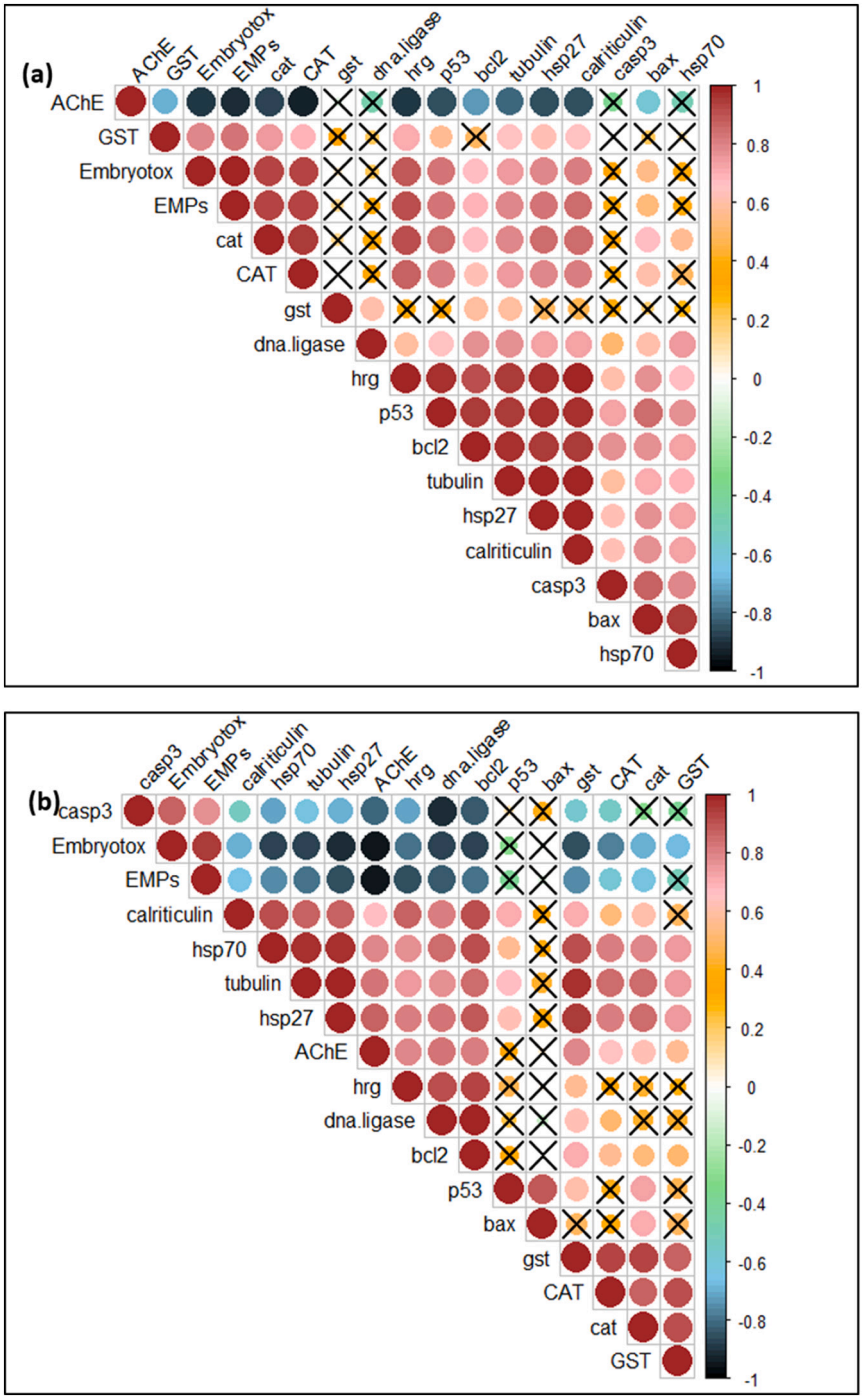

Fig. 7. Pearson Correlation matrix of multiple parameters in mussel D-larvae exposed to different concentrations of EMPs and salinity levels. Embryotox (Embryotoxicity), EMPs (EMPs uptake), Catalase activity (CAT), AChE activity (AChE), GST Activity (GST), genes' expression (cat, gst, hrg, tubulin, casp3, p53, dna ligase, bax, bcl2, hsp27, hsp70, and calreticulin).

mineralization, contributing to mussel larvae shell development (Abebe et al., 2007; Sforzini et al., 2020). Furthermore, tubulin in bivalve mollusks plays a role in the formation or shaping of different types of calcium carbonate polymorphs, potentially impacting the composition and structure of the shell of mussel larvae (De Wit et al., 2018). The positive regulation of hrg and tubulin has been documented in mussel D-Larvae under thermal stress (Mlouka et al., 2019). Therefore, the pivotal roles played by HRG and tubulin in shell biomineralization are likely to be key adaptation mechanisms of *Mytilus* sp. to the changing environment. The established immune function of HRG in adult mussels (Sforzini et al., 2020) suggests a potential involvement of HRG in immune-related processes in mussel larvae. Although the mussel immune system demonstrates sensitivity to nanoplastics (NPs) (Auguste et al., 2020), a comprehensive understanding of the biological effects of NPs requires a thorough understanding of their molecular interactions with cells in physiological environments. While in mammals, NPs form a 'protein corona' influencing their cellular interactions (Kopatz et al., 2023), these interactions remain poorly understood in the biological fluids of aquatic organisms. Generally, the presence of proteins reduces the surface energy of NPs through non-specific adsorption, resulting in decreased membrane adhesion and absorption efficiency, thereby being considered a protective effect against the potential cytotoxicity of

nanoparticles (Lesniak et al., 2013). (Canesi et al., 2016) demonstrated for the first time the formation of a protein corona in *M. galloprovincialis* mussels exposed to PS-NH2. The results of this study identified HRG (also known as MgC1q6) as the unique protein component of the corona around PS-NH2. In this study, the increased positive regulation of HRG following exposure to EMP may be associated with a potential formation of a protein corona in mussel larvae, implying a protective effect against the potential cytotoxicity of ingested EMPs <1.22 μm.

As physiological stress-related genes, intracellular HSP27 and HSP70 can contribute to the folding or degradation of stress-damaged proteins, thereby safeguarding cells against potentially lethal damage caused by environmental stress. Additionally, HSP27 and HSP70 can enhance their expression to cope with oxidative stress (Garrido et al., 2003). The exposure of mussel D-larvae to EMPs, either alone or combined with high salinity (37p.s.u), positively stimulates the expression of the hsp27 gene in mussel D-larvae. Nonetheless, this impact is moderately diminished when exposed to a salinity of 37p.s.u. This suggests that heightened salinity could potentially impede the cellular capacity to effectively cope with increased stress and alleviate the toxic effects encountered by mussel larvae. Concerning the hsp70 gene, high salinity (37p.s.u) markedly decreased the expression of the hsp70 gene in larvae exposed to 50 and 100 μg/L compared to 33p.s.u. These findings suggest that the genes hsp27 and hsp70 are involved in regulating the adaptation of mussels to high salinity. El-Leithy et al. (2019) revealed that the expression of hsp 27 and hsp70 genes was significantly altered under low and high salinity conditions in the gills and liver of the Nile tilapia *Oreochromis niloticus*.

Catalase, as an essential antioxidant enzyme, facilitates the conversion of hydrogen peroxide (H_2O_2) into water (H_2O) and molecular oxygen (O_2). Additionally, GST, a crucial enzyme, supports cellular detoxification by binding glutathione to harmful substances like peroxides, diminishing ROS. This enzymatic action converts peroxides such as H_2O_2 into less harmful compounds, reducing cellular oxidative stress. The exposure to EMPs under normal salinity conditions resulted in a positive upregulation of the cat gene expression. Recent findings have suggested that exposure to microplastics alone may lead to an imbalance in the intracellular defense system against reactive oxygen species (Qiao et al., 2019). Moreover, the concurrent exposure to high salinity (37p.s.u) alone or with low concentrations of EMPs resulted in an amplified upregulation of the cat and gst genes in D-larvae. This observation indicates that high salinity (37p.s.u) might enhance the toxic effects induced by EMPs on mussel D-larvae. It has been shown that brief fluctuations in salinity serve as robust activators of oxidative stress in the Mediterranean mussel (Gostyukhina et al., 2023), with higher salinity levels causing a more pronounced oxidative stress than lower salinity levels. The modulation of oxidative stress was confirmed at the biochemical level by assessing the enzymatic activities of CAT and GST. Our results indicated that oxidative stress induced by EMPs was detectable in mussel D larvae under normal salinity conditions (33p.s.u), as indicated by a rise in CAT and GST activities in line with the concentration applied. Li et al. (2022) demonstrated that MPs can induce oxidative stress in bivalves, as evidenced by an increase in CAT and GST activity. Moreover, fish exposed to EMPs show elevated CAT and GST activities compared to the control group throughout the exposure periods, indicating an alteration in enzymatic defense mechanisms in early-juvenile *Dicentrarchus labrax* following short-term EMP exposure (Zitouni et al., 2021). At high salinity (37p.s.u), CAT and GST activities in mussels D-larvae were significantly greater than that at 33p.s.u. It has been found that fluctuations in environmental salinity influence oxidative stress in aquatic organisms (Bal et al., 2021), including mussels (Freitas et al., 2017). Simultaneous exposure to low concentrations of EMP and high salinity (37p.s.u) triggers increased CAT and GST activities. Conversely, at higher concentrations, antioxidant activities are notably suppressed, suggesting that larvae lose their ability to cope with intensified multiple stressors.

The consequences of MPs ingestion can impact the nervous system by causing damage or altering the production rate of neurotransmitters. Due to its significant role in transmitting nerve impulses in cholinergic neurons, AChE is highly sensitive to both biotic and abiotic changes. Its inhibition is commonly employed as a biomarker to detect and measure neurotoxicity (Tlili et al., 2020). Neurotoxic effects of MPs, as indicated by AChE inhibition, have been observed in the adult stages of various bivalves. For instance, *Scrobicularia plana* exposed to polystyrene microparticles (20 mm) showed such effects (Ribeiro et al., 2017). Similarly, the mussel *M. galloprovincialis* displayed AChE inhibition when exposed to MPs associated with pyrene. Even the freshwater bivalve *Corbicula fluminea* exhibited AChE inhibition when exposed to MPs at a concentration of 0.13 mg/L (Oliveira et al., 2018). However, our study represents the first investigation focusing on the neurotoxic effects of EMPs on *M. galloprovincialis* larval stages. Our data demonstrate that exposing D-larvae to increasing concentrations of EMPs induces inhibition of AChE activity, providing evidence of neurotoxic effects caused by MPs during the early life stages of *M. galloprovincialis*.

5. Conclusion

Our study represents the first assessment of the combined effect of EMPs and salinity on the early developmental stages of mussels, providing compelling evidence regarding the significant threat posed by this combination to these vulnerable stages. Our observations revealed that elevated salinity leads to a decrease in EMP absorption by mussel D-larvae. This decline could be attributed to alterations in filtration rates, and feeding efficiency, as well as a reduced capacity of mussel larvae to capture and ingest particles in response to abnormal salinity conditions. We have shed light on the mechanism underlying the damage induced in mussel D-larvae by the combined or individual effects of EMPs and elevated salinity. Our findings reveal that exposure to this multi-stress leads to an increase in larval anomalies and developmental arrest rates. The effects on larval development were explored using a transcriptomic study highlighting the potential impacts of EMPs on shell biomineralization, indicating that HRG and tubulin are likely to be key adaptation mechanisms of *Mytilus* sp. to the changing environment. Additionally, the increased production of ROS coupled with the inadequate functioning of the antioxidant system induces oxidative stress, leading to DNA damage. Ultimately, this sequence of events results in the arrest of larval development through the activation of apoptosis. Nevertheless, the data highlight the vulnerability of early life stages of bivalves to salinity variation due to climate change and EMPs, providing baseline information for future research investigating their impact on marine ecosystems.

CRediT authorship contribution statement

Khouloud Boukadida: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Rania Mlouka:** Writing – original draft, Investigation, Formal analysis, Data curation. **Mohamed Rida Abelouah:** Formal analysis, Investigation, Methodology, Writing – original draft. **Souha Chelly:** Formal analysis, Data curation. **Ilef Romdhani:** Data curation, Formal analysis. **Gea Oliveri Conti:** Writing – review & editing, Methodology, Conceptualization. **Margherita Ferrante:** Writing – review & editing, Conceptualization, Methodology. **Matteo Cammarata:** Writing – review & editing, Methodology, Conceptualization. **Maria Giovanna Parisi:** Writing – review & editing, Conceptualization. **Aicha AitAlla:** Methodology, Funding acquisition, Conceptualization. **Mohamed Banni:** Writing – review & editing, Writing – original draft, Funding acquisition, Data curation, 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.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.172177.

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