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Adverse effects of environmentally relevant concentration of microplastics on gill epithelium permeability in the euryhaline Mediterranean killifish *Aphanius fasciatus*

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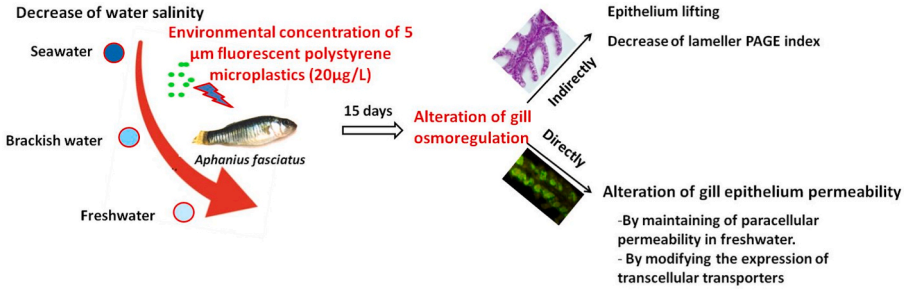
HIGHLIGHTS

- Effects of microplastics on gill osmoregulation were assayed in killifish.
- Microplastics alter gill osmoregulation function, particularly in freshwater.
- Microplastics alter both paracellular and transcellular permeability.

GRAPHICAL ABSTRACT



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ABSTRACT

Estuaries and lagoons are characterized by fluctuating salinity and significant amounts of microplastics (MPs) and are increasingly subjected to various anthropogenic pressures. We investigated whether the accumulation of MPs in the gills of fish inhabiting these fragile ecosystems alters osmoregulation and, consequently, their ability to tolerate fluctuating salinity. The effects of a 15-day exposure to an environmentally relevant concentration (20 µg/L) of spherical polystyrene microplastics (PS-MPs) with a diameter of 5 µm were assessed in the Mediterranean killifish *Aphanius fasciatus*, focusing on tissue and gene expression changes related to factors of paracellular and transcellular permeability of the gill epithelium during the transition from seawater to freshwater. Our results revealed that PS-MPs indirectly impaired osmoregulation, particularly in fresh water, through their toxic effects on the gill tissue. Toxicity was evidenced by epithelial lifting, a decrease in the proportion of secondary lamellae available for gas exchange, and upregulation of superoxide dismutase and heat shock protein

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genes. Furthermore, exposure to PS-MPs directly affected gill epithelial permeability by maintaining relatively high paracellular permeability through the downregulation of claudin 3 and by modifying the expression of the transcellular transporter Na^+/K^+-ATPase and cystic fibrosis transmembrane conductance regulator in the gill epithelium. Overall, these findings confirm the toxic effects of PS-MPs on gill tissue and demonstrate, for the first time, that environmentally relevant concentrations of MPs adversely affect gill epithelium permeability during decreased salinity acclimation in the euryhaline fish *A. fasciatus*.

1. Introduction

Plastic debris are the most abundant pollutants in aquatic ecosystems because of their high production rates, low recycling rates, and slow degradation of most plastic polymers (Garcés-Ordóñez et al., 2022). Microplastics (MPs), defined as plastic particles with a diameter of less than 5 mm, are emerging pollutants in aquatic environments. MPs can originate directly from industrial processes (primary MPs) or from the degradation of large plastic debris via various physicochemical processes (secondary MPs) (Weinstein et al., 2016). Polystyrene (PS), a polymer derived from styrene monomers, is widely used in the production of polystyrene foam, toys, medical equipment, cup covers, construction materials, fishing gear, packaging foam, food containers, and many other applications (Ho et al., 2018; Siddiqui et al., 2023). PS is one of the major types of MPs (PS-MPs) found in aquatic environments. Owing to its high abundance in sediments and water, it has become the subject of numerous studies (Bagheri et al., 2020). In these environments, PS-MPs arise primarily from the degradation of large plastic debris rather than from primary sources (Badylak et al., 2021).

The highest concentrations of MPs have been detected in shallow coastal waters, such as estuaries and lagoons, which are surrounded by densely populated coastlines and are increasingly subjected to various anthropogenic pressures, including chemical pollution (Iñiguez et al., 2016). A seasonal evaluation of floating MPs in a shallow Mediterranean coastal lagoon by Simantiris et al. (2022) reported an average of 40–50 particles/L, with a particular abundance of nylon and polyester fibers. A recent analysis of MPs in estuaries along the Arabian Sea coast detected higher concentrations, ranging from 100 to 650 particles/L (Sunil et al., 2024). These transitional waters are fragile ecosystems with significant ecological importance, providing feeding resources and habitats for various plant and animal species (Costanza et al., 1997; Esteves et al., 2008). The presence of sediment traps and low water exchange are natural characteristics of lagoons and estuaries that favor the accumulation of MPs (Martellini et al., 2018). Consequently, accumulated MPs can negatively affect the physiology and ecology of animals in these environments (Lusher et al., 2017). Several *in situ* and *in vivo* studies have shown that ingested MPs accumulate in fish tissues (Garcés-Ordonez et al., 2022) and disrupt parameters related to growth, reproduction (Cormier et al., 2021), immunity (Li et al., 2024), and metabolism (Wang et al., 2022). In fish from lagoons and estuarine environments, the highest levels of MPs have been detected in the intestines and gills (Su et al., 2019). However, their effects on gill function at environmentally realistic concentrations remain unclear.

Estuaries and coastal lagoons are characterized by highly fluctuating salinity and are inhabited by euryhaline fish, which can adapt their osmoregulation according to the salinity of the external environment (Kültz, 2015). The gill, which is in direct contact with the surrounding water, must exhibit extreme plasticity to cope with fluctuating salinity (Evans et al., 2005). Under external hypo-osmolarity conditions, the gill epithelium is crucial for maintaining the hydromineral balance by actively acquiring ions from the surrounding water through transcellular ion transport and restricting obligatory passive ion loss through the paracellular pathway between gill epithelial cells via the gill tight junction (TJ) complex (Kültz, 2015). Responding to changes in salinity is extremely stressful for fish and can be challenging because they must counteract solute gradients using active mechanisms to maintain a constant internal environment (Kültz, 2015). For instance, the metabolic energy required for osmoregulation and the transition from seawater (SW) to freshwater (FW) is energetically expensive, costing killifish up to 10% of their total energy budget (Kidder et al., 2006), which constitutes a significant pressure for the evolution of "behavioral osmoregulation'' in this estuarine species (Kidder et al., 2006). We hypothesized that the ingestion of MPs and their accumulation in the gills represent an additional challenge for fish to cope with fluctuating salinity in lagoon and estuarine waters, potentially affecting the capacity of the gill epithelium to maintain an adequate hydromineral balance.

The Mediterranean killifish, *Aphanius fasciatus*, is found along the coastal zones of the central and eastern Mediterranean regions. It is one of the most strictly estuarine- and lagoon-dependent fish species (Whitehead et al., 1986). *A. fasciatus* is recognized as one of the most euryhaline species in the Mediterranean Sea and can live and reproduce across a broad range of salinities (Leonardos and Sinis, 1998). Several studies have highlighted the suitability of *A. fasciatus* as an indicator species for transitional water pollution by using its molecular, cellular, and physiological responses to elucidate the effects of pollutants on resident organisms in estuaries and lagoons (Messaoudi et al., 2009; Kessabi et al., 2010; Lionetto et al., 2023). Unfortunately, populations of *A. fasciatus* have dramatically declined due to pollution (Leonardo, 2008), leading to its listing in annexes II and III of the "Bern Convention, " which pertains to the conservation of wildlife and the natural environment in Europe (Council of Europe, 2000). In a recent study, we demonstrated that exposure of *A. fasciatus* to environmental MPs results in significant histological alterations and oxidative stress in the gills (Kessabi et al., 2023). However, it remains unknown whether MPs affect the gill function of osmoregulation and, consequently, the ability of this euryhaline species to adapt to fluctuating salinity in its habitat. Therefore, in this study, we aimed to assess the tissue and gene expression profiles of certain factors related to paracellular and transcellular permeability of the gill epithelium in *A. fasciatus* in response to the transition from SW to FW. Our results will contribute to the understanding of the impact of MPs on gill function and may help to elucidate one of the potential causes of the decline in *A. fasciatus* populations.

2. Materials and methods

2.1. Polystyrene microplastic particles (PS-MPs)

To assess the distribution and accumulation of MPs in the gills of killifish, we used spherical fluorescent polystyrene (PS-MPs) particles with 5 μm diameter (PS-FluoGreen-Fi199; GmbH, Berlin, Germany). The particles had excitation and emission wavelengths at 502 and 518 nm, respectively (Fig. 1). The assessment of the PS spheres revealed an average particle agglomerate size of 4.98 μm ± 0.15, and a ζ-potential of −0.0934 ± 0.172. In this study, we used standard spherical PS-MPs instead of environmental MPs to limit factors that could interfere with gill epithelium permeability, such as the shape, size, and chemical nature of the polymers, which could complicate result interpretation. This spherical and pristine form of MP was also used to characterize only the potential deleterious effects of PS on gill epithelium permeability, independent of all compounds that may adhere to the surface of the particles under environmental conditions (e.g., trace metals, organic pollutants, and pathogenic germs).

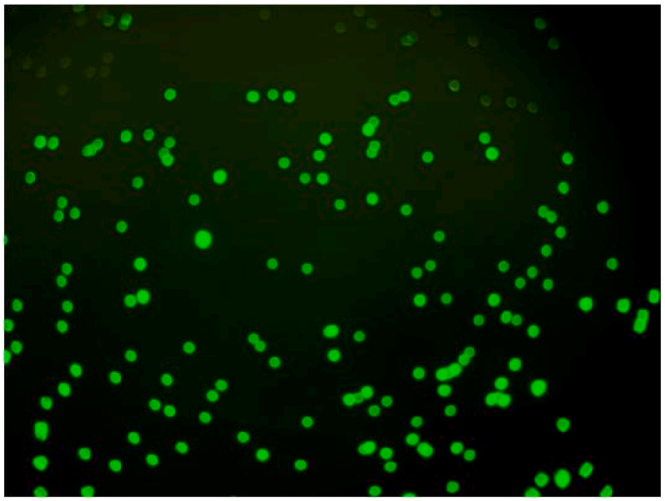

Fig. 1. Spherical fluorescent polystyrene (PS-MPs) particles with a diameter of 5 μm (PS-FluoGreen-Fi199; GmbH, Berlin, Germany). The excitation and emission wavelengths were 502 and 518 nm, respectively. Gr: × 100.

2.2. Animals and experimental design

A. fasciatus juveniles that were sexually immature and weighed in average 0.38 ± 0.05 g were collected from a coastal area in Monastir, Tunisia, in October 2022 by professional fishermen using a 4-m beam trawl. The selection of juvenile fish as a study model was based on bibliographic data, indicating the relevance of this developmental stage in assessing MPs contamination in estuarine fish (Kazour et al., 2020). Our previous study highlighted the suitability of histological and molecular responses of juvenile *A. fasciatus* gills to MP exposure (Kessabi et al., 2023). Fish were transported alive to the laboratory and housed in 150 L glass aquaria with SW under conditions similar to the collection sites: temperature, 16 °C; photoperiod, 12 h light/12 h dark; salinity, 33 ppt; and dissolved oxygen, 6.2 ± 0.6 mg/L. Fish were fed once daily with commercially balanced fish food sticks (Tetramine, Hagen, France). The medium was renewed every 48 h, and inspections were conducted twice daily to remove wounded, diseased, or dead fish. Prior to experimentation, each fish was acclimated to the laboratory for one week under these conditions.

As shown in Fig. 2, after the acclimation period, one batch of fish remained in **SW** to form a control group (**C-SW**) and an exposed group to PS-MP (**MP-SW**) at a concentration of 20 μg/L for two weeks. Another batch was acclimated for 24 h to brackish water (**BW**) obtained by adding local fresh tap water (**FW**) to **SW** (1:1, v/v) (Seo et al., 2009; Chandrasekar et al., 2014). Subsequently, some BW fish were divided into a control group (**C-BW**) and PS-MP-exposed group (**MP-BW**). The remaining fish were transferred directly to **FW** and divided into a control group (**C-FW**) and PS-MP-exposed group (**MP-FW**). Both the MP-FW and MP-BW groups were maintained under the same conditions as the MP-SW group. Data relating to the different animal groups and protocol conditions are summarized in Table 1. According to several published studies, the concentration of 20 μg/L PS-MP used in this study was within the environmentally relevant levels (Dubaish and Liebezeit, 2013; Zhao et al., 2014; Bergmann et al., 2017). The facilities and animal use protocols for fish were reviewed and approved by the local ethics committee of the Institute of Biotechnology, University of Monastir, Tunisia (protocol number: CERSVS/ISBM038/2022). Animal housing adhered to the EEC 609/86 directives, which regulate the care of the experimental animals. After two weeks of PS-MP exposure, the animals were weighed and euthanized by decapitation. For histological and immunofluorescence analyses, the gills were removed and preserved in 10% neutral-buffered formalin. One gill filament per fish was collected for RNA extraction and later molecular studies. Gill filaments intended for Na^+/K^+-ATPase activity were stored at −80 °C.

2.3. Analytical procedures

2.3.1. Na^+/K^+ ATPase activity

Na^+/K^+-ATPase activity in the gill filaments was determined using the method described by McCormick (1993). This activity is based on

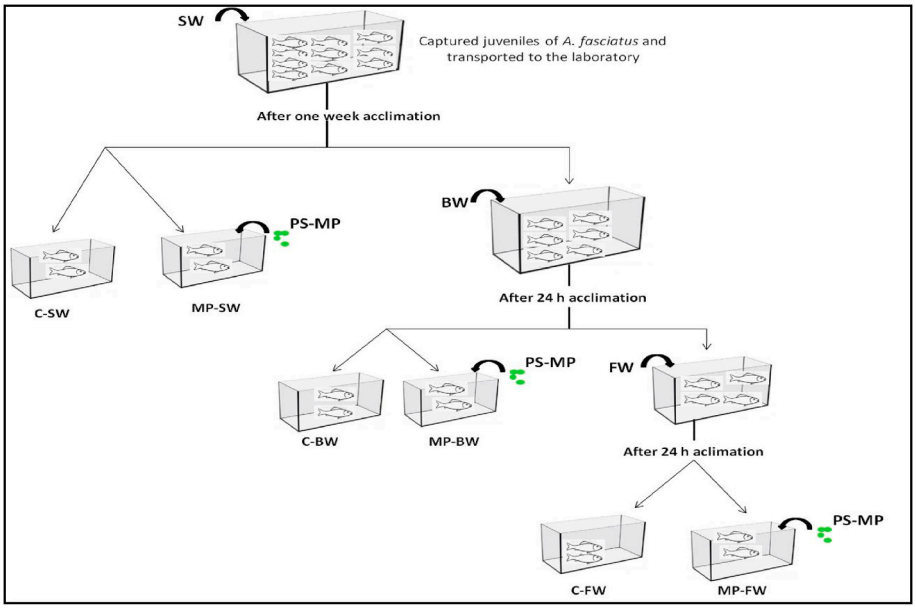

Fig. 2. Experimental design: Captured juveniles of *Aphanius fasciatus* (***A. fasciatus***) were maintained in the laboratory in seawater (**SW**) under conditions similar to those of the collection sites. After one week of acclimation, some fish were transferred to two 15 L glass aquaria without (**C-SW**) or with fluorescent polystyrene microplastics (**MP-SW**), and the remaining fish were transferred to 70 L glass aquaria containing brackish water (**BW**). After 24 h of acclimation in BW, some fish were transferred to two 15 L glass aquaria without (**C-BW**) or with fluorescent polystyrene microplastics (**MP-BW**), and the remaining fish were transferred to 50 L glass aquaria containing freshwater (**FW**). After 24 h of acclimation to FW, some fish were transferred to two 15 L glass aquaria without (**C-FW**) or with fluorescent polystyrene microplastics (**MP-FW**). Fish transferred to 15 L aquaria were sacrificed after 15 days.

Table 1
Fish and water parameters in different conditions.

Conditions		C-SW	MP-SW	C-BW	MP-BW	C-FW	MP-FW
Fish	Number	30	30	30	30	30	30
	Length (cm)	2.88 ± 0.29	2.97 ± 0.30	2.73 ± 0.11	2.87 ± 0.29	2.90 ± 0.25	2.63 ± 0.14
	Weight (g)	0.45 ± 0.09	0.41 ± 0.04	0.34 ± 0.06	0.35 ± 0.09	0.44 ± 0.05	0.32 ± 0.06
Water	Salinity (ppt)	33.00	33.00	16.00	16.00	0.94	0.94
	Osmolality (mOsm/kg)	555.00	555.00	285.00	285.00	31.80	31.80
	Na^+ mmol/L	1430.00	1430.00	737.00	737.00	41.01	41.01
	Cl^- mmol/L	930.00	930.00	478.00	478.00	26.56	26.56

the sensitivity of the enzyme to ouabaïn, and is detected through the enzymatic coupling of ATP dephosphorylation to NADH oxidation.

2.3.2. Histological analysis

After being submerged in progressively higher concentrations of ethanol for dehydration, the fixed gills were hyalinized in Ottix Plus and Ottix Shaper, and then embedded in paraffin wax at 56 °C. Periodic acid-Schiff staining was applied to all specimens in 4 μm sections to reveal the branchial structure and mucus. Slides were examined at 40 × and 100 × magnification under an optical microscope (Leica DM200, Wetzlar, Germany), and micrographs were captured using a digital camera (Leica ICC50W, Wetzlar, Germany) with the Las EZ 3.4 software.

The secondary lamellar length (SLL), basal epithelial thickness (BET), and inter-lamellar distance (ID) are shown in Fig. 3. Three measurements were performed for each filament (distal, central, and proximal regions). The proportion of secondary lamellae available for gas exchange (PAGE index) was calculated using the formula described by Nero et al. (2006): PAGE index (%) = 100 × [SLL/(BET + SLL)]. The PAGE index correlates with the total gas exchange surface area of the fish gills (Don Stevens, 1992).

2.3.3. Immunofluorescence

For Na^+/K^+-ATPase, sodium-chloride exchanger (SLCA12/NKCC2), and cystic fibrosis transmembrane conductance regulator (CFTR), 4 μm sections of gills were deparaffinized, rehydrated, and processed as previously described (Pariante et al., 2016). Briefly, antigen retrieval was performed under pressure by heating the slides at 90 °C for 30 min. Non-specific sites were saturated before the addition of Na^+/K^+-ATPase (ab195884), SLCA12/NKCC2 (ab244342), CFTR (ab131553), Cldn 3 (ab15102), or Cldn 4 (ab53156) antibodies (Abcam, Cambridge, UK) diluted 1:100 for overnight incubation at 4 °C. The slides were then incubated for 1 h with the appropriate goat anti-rabbit secondary antibody (Alexa Fluor 488,1:300 dilution). Images were captured using an epifluorescence microscope (Zeiss AxiostarPlus, Germany). The levels of Na^+/K^+-ATPase, SLCA12/NKCC2, CFTR, Cldn 3, and Cldn 4 were estimated by quantifying the green fluorescence intensity using the ImageJ software. Additionally, some slides were observed at 488 nm without antibody staining to confirm the fixation of PS-MPs on to the gill tissue.

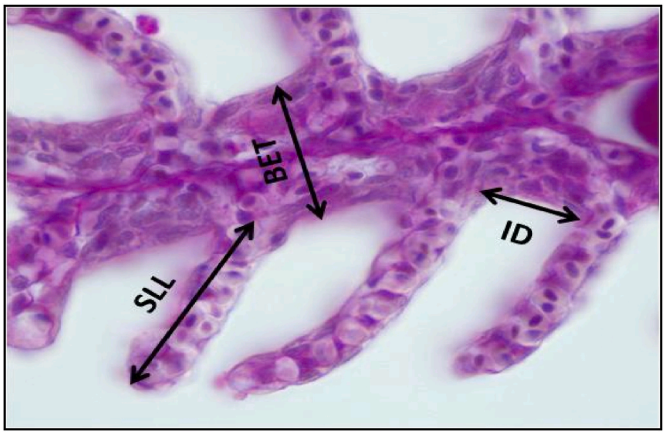

Fig. 3. Measurements of the gill filament of *Aphanius fasciatus* were used to estimate the proportion of secondary lamellae available for gas exchange (**PAGE index**).
SLL: secondary lamellar length; ID: interlamellar distance; BET: basal epithelial thickness. Periodic acid-Schiff staining, Gr: × 40.

2.3.4. Molecular analysis

2.3.4.1. Sequence analysis and primer design. The DNA sequences of Na^+/K^+-ATPase, CFTR, NKCC2, Cldn3, and Cldn4 were obtained using the SRS tool from the European Bioinformatics Institute (http://www.ebi.ac.uk/). Conserved sequence regions were identified by aligning known Cyprinodonta sequences when available, and supplemented with Percomorpha sequences, as required, using the EMBOSS EMMA tool (Rice et al., 2000). Gene-specific primer sets were then designed from these consensus sequences using Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA).

2.3.4.2. RNA extraction, sequencing, and quantitative real-time PCR analysis. Total RNA was extracted from the gill tissues using Trizol® reagent (ThermoFisher Scientific, Waltham, MA, USA). RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific), and RNA quality was assessed using a2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). To prevent genomic DNA contamination, RNA was treated with DNase I. Primers for the target genes (NKA, CFTR, NKCC2, Cldn3, and Cldn4) were designed based on sequences from other cyprinodont species to amplify the corresponding cDNA fragments. The PCR amplicons were sequenced using a3730 DNA Analyzer (Applied Biosystems, Foster City, California), and the BLAST algorithm on the NCBI server was used to search for homologous genes in GenBank (primer sequences are listed in Table 2). Sequences from at least three independent clones were deposited in the GenBank database. The First Strand cDNA Synthesis Kit (Roche) was used to reverse transcribe DNase I-treated RNA (100 ng–1 μg) into cDNA, which was stored at −20 °C. For the quantification of specific transcripts, 100 ng of total RNA was used in a Light Cycler® 480 Real-Time PCR System (Roche) using SYBR® Green Mix (Takara Bio, Kusatsu, Shiga, Japan) and specific primer pairs (Table 2). The second derivative maximum (Cp) was used to quantify the relative mRNA levels of the stress-related genes, which were measured in triplicate. ΔCp values (ΔCp = Cpref - Cptg) were calculated by comparing the Cp values of the target genes to a reference gene, β-actin, which was selected after assessing its stability across all samples (Pfaffl, 2001). Primer adjustments were performed as required. mRNA abundance values were expressed as target gene mRNA copies per 1000 reference gene mRNA copies (‰ of the reference gene, $1000 \times 2^{\Delta Cp}$).

2.3.5. Statistical analysis

All data are presented as the mean ± standard error. Differences were considered statistically significant at $p < 0.05$. Statistical analysis was performed using one-way analysis of variance, followed by Tukey's post hoc *t*-test and paired tests, where appropriate, using GraphPad Prism 9.0.

For molecular studies, statistical analyses were performed using the

Table 2
QRT-PCR primers of the corresponding *Aphanius fasciatus* sequences developed in this work.

Gene	Acronym	Forward primer (5′-3′)	Reverse primer (5′-3′)
Na^+/K^+-ATPase Pump	Na^+/K^+-ATPase	CCTCTTCATCATCGCCAACA	GATGTCGCTCTCAGCAGCTTC
CysticFibrosisTransmembraneRegulator	CFTR	GCATACTGGCCCTTGGAGTC	CCCGTAGCACAGAGCTAAGCA
$Na^+/K^+/2Cl^-$ Pump	NKCC	AAGAAGAGGTGGAAGGACTGCA	GCGGTCATGATCAATCCTGTT
Claudine 3	Cldn3	ACTGGTTCCTGTTCAAAGAGTGG	CACCATCGCGTAGAGGCC
Claudine 4	Cldn4	ATGACGGACCTGAAGGAGGG	TTGTCYCGGTCGGCGAAG
Heatshockprotein 70	HSP70	TTTTATCCTGGTGGTGGC	GCCCTCTTGTTGTCTCTGAT
Superoxidedismutase	SOD	AGAGCATGGTTTCCATGTCCAT	CCAACTCACCAGGTCTCCAA

SPSS software (version 17, SPSS Inc., Chicago, IL, USA). All calculations were based on ΔCp values, which were confirmed to follow normal distributions according to the Kolmogorov–Smirnov test.

3. Results

3.1. PS-MPs accumulation in gill tissue

The accumulation of PS-MPs in the gill tissue is shown in Fig. 4. The accumulated PS-MPs appeared as spherical fluorescent points distributed throughout the gill tissue. PS-MPs primarily accumulated in the primary lamellae, secondary lamellae, and ionocytes. The tissue distribution did not differ with variations in salinity, and was similar across the different groups of fish exposed to MPs.

3.2. PS-MPs effects on gill tissue

Similar to other teleosts, the gill tissue of *A. fasciatus* is composed of filaments, each containing a primary lamella and secondary lamellae, with an epithelium comprising various cell types, including ionocytes, pavement cells, and mucus cells (MCs). Ionocytes were primarily located in the epithelium of the primary lamellae of fish in all groups. In fish from the C-SW group, ionocytes were associated with accessory cells (ACs) and were more abundant than in fish from the C-BW and C-FW groups (Fig. 5A). The density of MCs and ACs, as well as the thickness of the gill epithelium, decreased with decreasing salinity. MCs and ACs were almost absent in fish from the C-FW group (Fig. 5A). A significant increase in the PAGE index ($p < 0.01$) was also noted in C-BW and C-FW fish compared with that in C-SW fish (Fig. 5B). Exposure to MPs did not induce remarkable modifications in gill histology; the toxic effects were limited to partial detachment of the lamellar epithelium, which was more pronounced in MP-BW fish and even more pronounced in MP-FW fish (Fig. 5A). Exposure to MPs also caused a significant decrease in the PAGE indices of BW ($p < 0.01$) and FW ($p < 0.05$) fish. At the molecular level, the toxicity of MPs in the gill tissue was manifested by a highly significant increase in the expression of the gene encoding the heat shock proteinHSP70in MP-FW fish (Fig. 5C), and the gene encoding superoxide dismutase (SOD) (Fig. 5D) in both MP-BW and MP-FW fish.

3.3. Epithelium permeability

The permeability of the gill epithelium was evaluated by assessing the tissue and gene expression of Na^+/K^+-ATPase, NKCC, and CFTR as factors of transcellular permeability, and the gene expression of claudin 3 and claudin 4 as factors of paracellular permeability.

3.3.1. Immunolocalization, activity, and gene expression of Na^+/K^+-ATPase

In the gills of SW fish, Na^+/K^+-ATPase immunofluorescence was primarily observed in the basolateral region of the primary lamellar ionocytes (Fig. 6A). Fluorescence remained localized in ionocytes in fish transferred to BW and FW, but significantly decreased in intensity with decreasing salinity (Fig. 6B). The highest Na^+/K^+-ATPase activity was also observed in the gills of fish in SW compared to those in BW and FW (Fig. 6C). Molecular analysis showed that the decrease in salinity had no effect on the expression of the gene encoding Na^+/K^+-ATPase (Fig. 6C). A significant decrease in the fluorescence intensity of Na^+/K^+-ATPase in the ionocytes of fish from BW and FW, compared to those from SW ($p < 0.05$ and $p < 0.01$, respectively), was observed under the effect of PS-MP (Fig. 6A and B). Exposure to PS-MP also significantly decreased the enzyme activity and gene expression in fish transferred to FW (Fig. 6C and D).

3.3.2. Immunolocalization and gene expression of NKCC

NKCC immunofluorescence was primarily localized in the basolateral region of ionocytes and was limited to the primary lamellae of the gills of fish in all groups (Fig. 7A). A highly significant decrease in fluorescence intensity ($p < 0.01$) was observed in fish from FW compared with those from SW and BW (Fig. 7B). FW ionocytes appeared larger than SW ionocytes, and NKCC expression was slightly apical. The decrease in salinity had no effect on the expression of the gene encoding NKCC, because the number of mRNA copies did not differ significantly among the fish in any of the groups (Fig. 7C). Under PS-MPs exposure, a significant decrease ($p < 0.05$) in the intensity of NKCC fluorescence was observed in fish in the MP-SW group (Fig. 7B) compared to that in the SW group (Fig. 6A). However, in freshwater, the effect of PS-MP exposure manifested as an increase in NKCC staining in the fish in the MP-FW group (Fig. 7B).

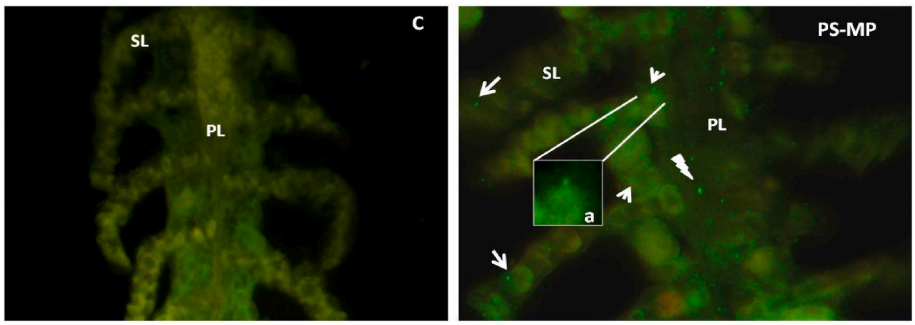

Fig. 4. Localization of 5 μm fluorescent polystyrene microplastics (**PS-MP**) in gill tissue.
SL: secondary lamellae; PL: primary lamellae; arrows: PS-MP in SL; arrowheads: PS-MP in ionocytes; PS-MP in PL. (Gr: × 40 (a: × 100)).

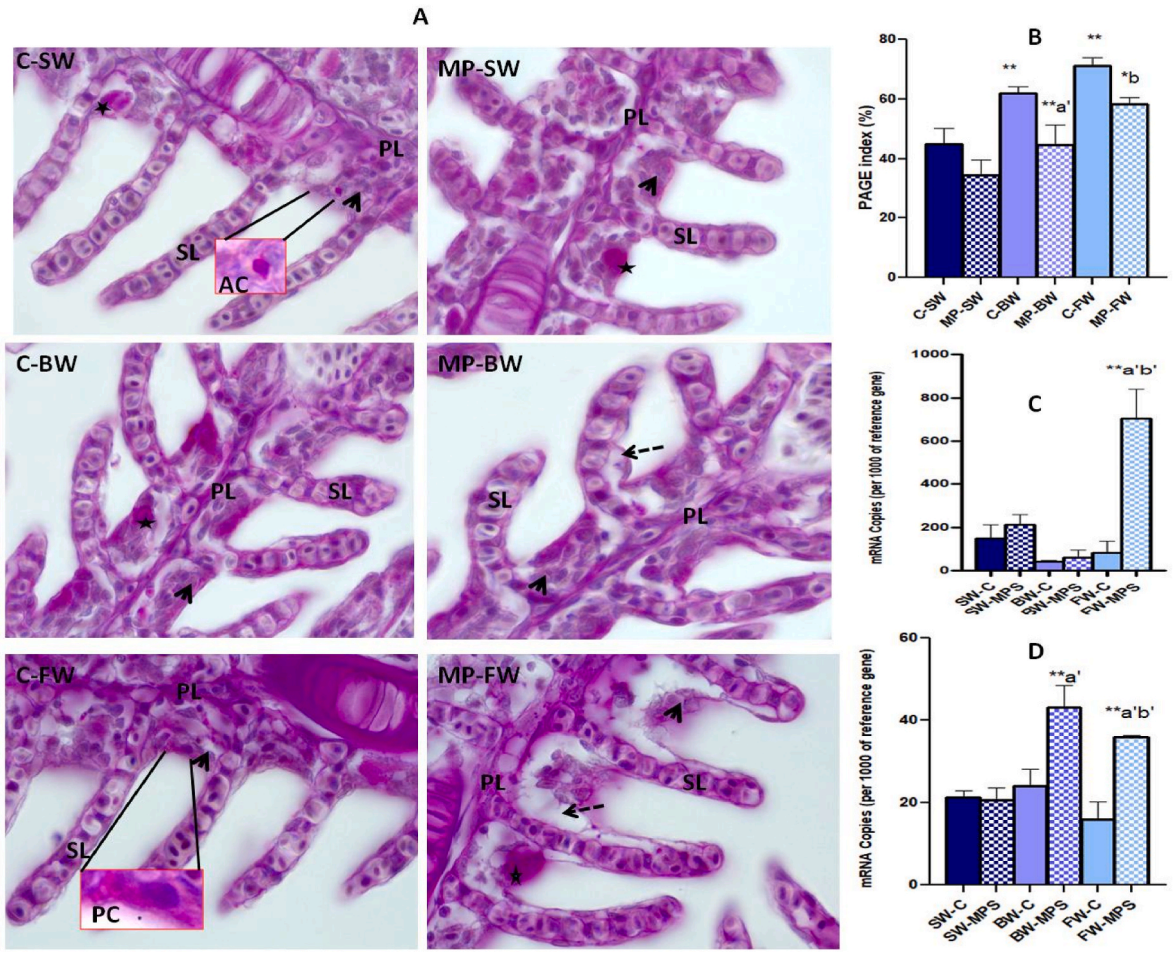

Fig. 5. Histology of gill filaments (**A**), PAGE index (**B**), and gene expression of heat shock proteins (HSP70) (**C**) and superoxide dismutase (SOD) (**D**) in gills of fish acclimated to **SW** and those transferred in **BW** and **FW** in the absence (**C-SW, C-BW, and C-FW**) or presence of polystyrene microplastics (**MP-SW, MP-BW, and MP-FW**).

AC: accessory cell; PC: pavement cell; SL: secondary lamellae; PL: primary lamellae; arrow head: ionocytes; arrow: detachment of the lamellar epithelium; star: mucus cell; *: $p < 0.05$ and **: 0.01 versus SW; a': $p < 0.01$ versus BW; b: $p < 0.05$ and b': $p < 0.01$ versus FW. (Gr: ×40; insets AC and PC: ×100).

3.3.3. *Immunolocalization and gene expression of CFTR*

Similar to Na^+/K^+-ATPase, CFTR was primarily located in the ionocytes of the primary lamellae, and the staining intensity decreased significantly with water salinity, reaching its lowest value in fish in the FW group (Fig. 8A and B). We also observed that CFTR distribution in ionocytes was both apical and subapical (Fig. 8A). Exposure to PS-MPs caused a significant decrease in the gill tissue expression of CFTR in MP-SW fish compared to that in the SW group. Conversely, in FW, PS-MPs increased the CFTR fluorescence intensity (Fig. 8A and B). The highest expression of the gene encoding CFTR was noted in the fish in the SW group, and the effect of exposure to PS-MP was manifested by a significant increase in the copy number of the corresponding mRNA in the MP-FW fish compared to that in the FW fish (Fig. 8C).

3.3.4. *Gene expression of claudin 3 and claudin 4*

Molecular analysis indicated that the expression of the gene encoding Cldn 4 did not differ significantly among fish from the different groups (Fig. 9B). In contrast, the Cldn 3 gene increased considerably with decreasing salinity, reaching its highest value in FW fish (Fig. 9A). A significant decrease in Cldn 3 gene ($p < 0.05$) expression was observed in fish in the MP-FW group compared to that in the FW group.

4. Discussion

Estuaries and lagoons are naturally characterized by fluctuating salinity and significant accumulation of MPs owing to sediment traps and slower water exchange. Fish inhabiting these transitional waters are euryhaline, and their gills exhibit extreme plasticity in coping with permanent salinity fluctuations. However, it remains unclear whether the increased accumulation of MPs in the gills of these fish, as described in several studies, alters their osmoregulatory function and, consequently, their ability to adapt to fluctuating salinity in fragile ecosystems. *A. fasciatus* is one of the most strictly estuarine and lagoon-dependent fish species, and was used as a model to study the tissue profile and gene expression of some factors of paracellular and transcellular permeability of the gill epithelium in response to decreased water salinity.

Gills are vital for respiration and osmoregulation in fishes. They are in continuous contact with chemical pollutants and particles, and are widely recognized as one of the most accumulated tissues for MPs (Karami et al., 2016; Limonta et al., 2019). Notably, the highest levels of accumulated MPs have been reported in the gills of euryhaline fish (Su et al., 2019). Using fluorescent PS-MPs, we successfully localized the particles in the gill filaments and ionocytes. Although the mechanisms involved in the translocation and internalization of PS-MPs across the cell membrane are poorly understood, some studies have confirmed that PS-MPs enter the cells by disrupting cell membrane integrity. Fleury and Baulinc (2021) reported that PS-MP beads (1–10 μm) attach to lipid membranes, leading to significant stretching of the lipid bilayer. Additionally, it has been demonstrated that interactions between nano and

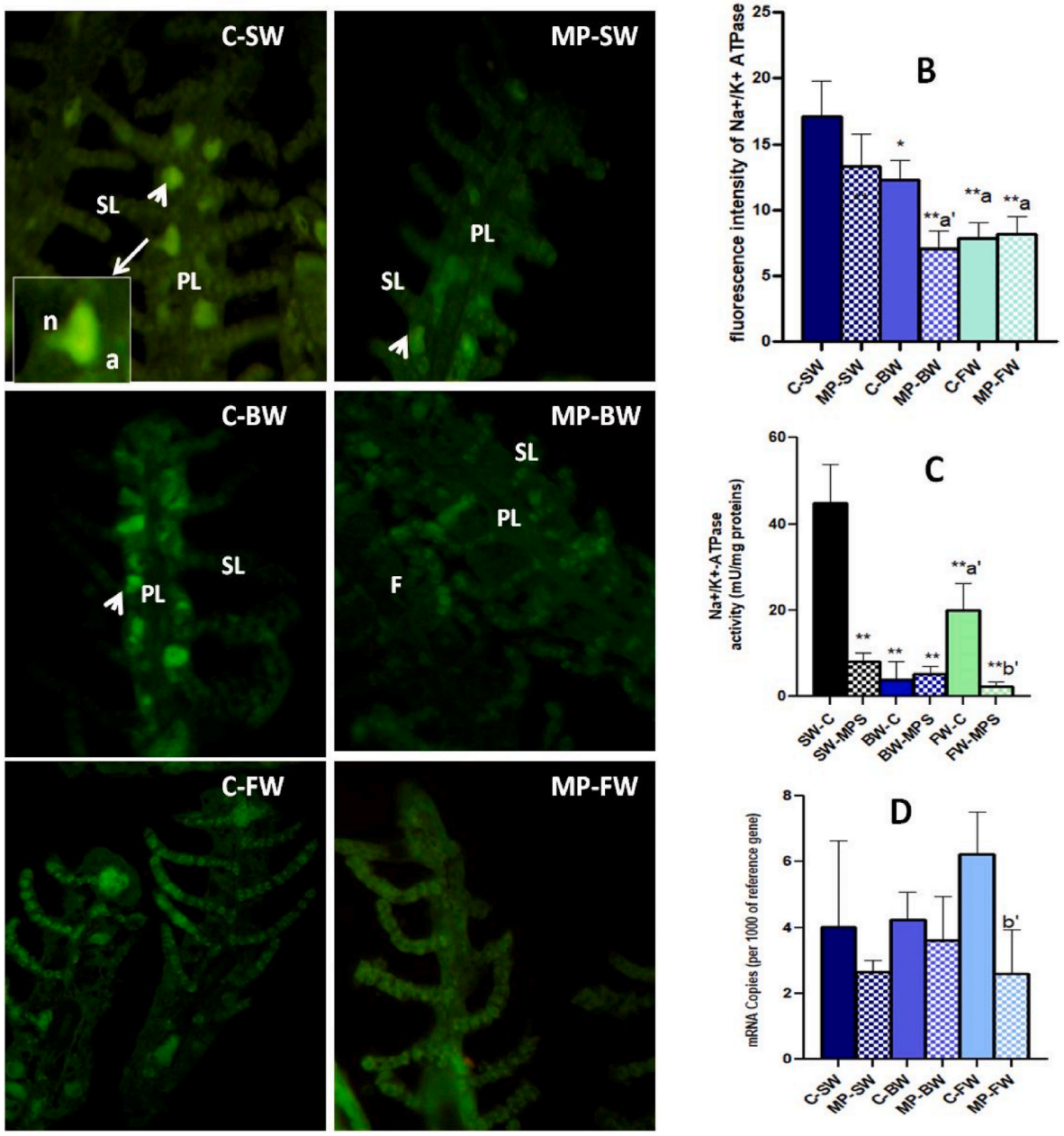

Fig. 6. Immunolocalization (**A**), fluorescence intensity (**B**), activity (**C**), and gene expression (**D**) of Na^+/K^+-ATPase in the gills of fish acclimated to seawater (**SW**) and those transferred in **BW** and **FW** in the absence (**C-SW, C-BW, and C-FW**) or presence of polystyrene microplastics (**MP-SW**, **MP-BW,** and **MP-FW**).
SL: secondary lamellae; PL: primary lamellae; arrow head: ionocytes; arrows: basolateral fluorescence of Na^+/K^+-ATPase in ionocyte; n: nuclei of ionocytes; *: $p < 0.05$ and **: 0.01 versus SW; a: $p < 0.05$ and a': $p < 0.01$ versus BW; b': $p < 0.01$ versus FW. (Gr:×40; inset "a": x 100).

micro PS (0.05–5 μm) and the cell membrane are mediated by hydrophobic and van der Waals forces, facilitating PS internalization (Liu et al., 2021). Other studies have shown that internalization of PS-MPs can occur without altering the cell membrane through endocytosis and macropinocytosis mechanisms; however, these internalization pathways have only been verified for PS smaller than 5 μm (Liu et al., 2021; Xu et al., 2021). It appears that water salinity does not affect the accumulation and distribution of PS-MPs in the gill tissue, but future quantitative analyses are needed to confirm this finding.

Although several studies have highlighted the toxicity of MPs in the gill tissues (Wang et al., 2019; Hu et al., 2020; Kessabi et al., 2023), it remains unclear whether this toxicity alters osmoregulation. Consistent with previous studies, we observed toxic effects of PS-MPs in the gill tissue, particularly in fish from the MP-FW group, including detachment of the lamellar epithelium, reduction in the PAGE index, and upregulation of genes coding for HSP70 and SOD. These alterations may indirectly indicate disruption of osmoregulation in response to decreasing salinity. Epithelial lifting and a decrease in the PAGE index have been considered by several authors (Rodrigues et al., 2015; Hu et al., 2020) as symptoms of osmoregulation disorders that could also affect respiratory gas exchange (Flores-Lopes and Thomaz, 2011). It is well established that the transcriptional responses of genes encoding stress proteins, such as HSP70 and SOD, can serve as useful biomarkers for biomonitoring aquatic environments in fish, particularly in *A. fasciatus* (Kessabi et al., 2010; Sinha et al., 2012). HSP70 levels can significantly increase following exposure to various environmental pollutants, including MPs (Abarghouei et al., 2021; Choi et al., 2023), as it is associated with various physiological processes and stress resistance in fish. The

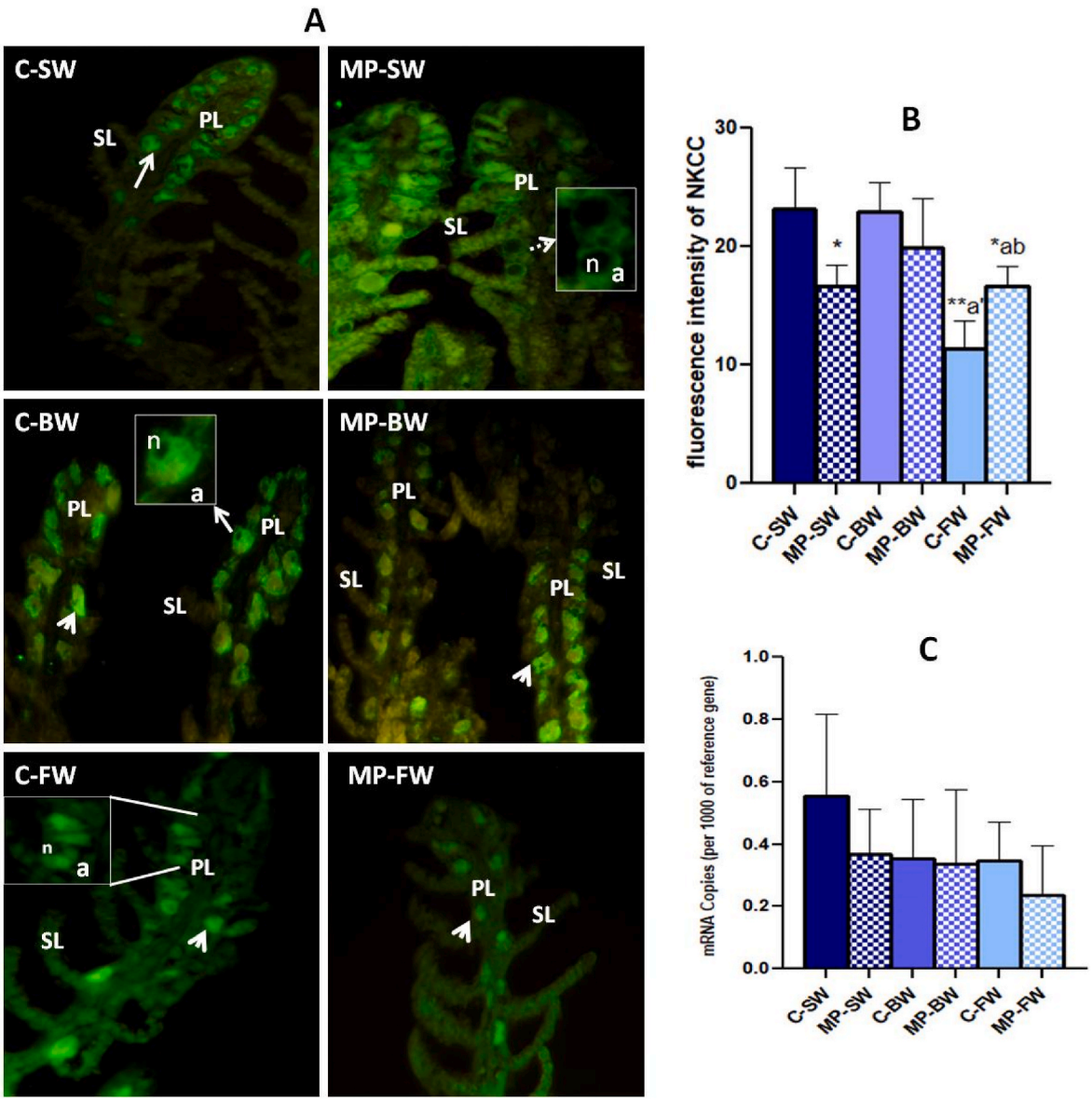

Fig. 7. Immunolocalization (**A**), fluorescence intensity (**B**), and gene expression (**C**) of $Na^+/K^+/2Cl-$ cotransporter (NKCC) in the gills of fish acclimated to seawater (**SW**) and those transferred in **BW** and **FW** in the absence (**C-SW, C-BW, and C-FW**) or presence of polystyrene microplastics (**MP-SW**, **MP-BW,** and **MP-FW**). SL: secondary lamellae; PL: primary lamellae; n: nuclei of ionocytes; arrow head: ionocytes; arrows: basolateral fluorescence of NKCC in ionocyte;: lack NKCC staining in ionocyte; *: $p < 0.05$ and **: 0.01 versus SW; a: $p < 0.05$ and a': $p < 0.01$ versus BW; b: $p < 0.05$versus FW(Gr:×40, insets "a": x 100).

significance of our results lies in the observation of a significant increase in HSP70 and SOD genes in the gills of fish from the FW and BW groups following exposure to environmentally realistic concentrations of PS-MP (20 μg/mL), compared to previous studies that used much higher concentrations of PS-MPs, such as 60 mg/L (Abarghouei et al., 2021) and 300 mg/L (Choi et al., 2023).

As noted by Zink and Wood (2024) in their recent prospective review, only four studies have focused on the direct effects of MPs on gill ionoregulation. This study is the first to evaluate the direct effects of exposure to environmentally relevant concentrations of PS-MPs on the transcellular and paracellular permeability of the gill epithelium in euryhaline fish, where osmoregulation is essential for adaptation to fluctuating salinity in its habitat.

In this study, the transcellular permeability was explored by comparing the tissue and gene expression profiles of the most important membrane transporters, Na^+/K^+-ATPase, NKCC, and CFTR, in response to decreased water salinity in the presence or absence of PS-MPs. Our results indicated that the tissue expression of Na^+/K^+-ATPase, NKCC, and CFTR transporters was primarily localized in primary lamellar ionocytes, with the highest values observed in SW treatments. The transfer of fish to lower salinity did not alter the localization of the studied transporters, but significantly reduced their tissue expression. It is well established that exposure to higher salinity increases the expression of Na^+/K^+-ATPase (McCormick, 2001), NKCC (Marshall et al., 2002; Katoh et al., 2008; Flemmer et al., 2010), and CFTR (Katoh et al., 2003; Shaw et al., 2008)in euryhaline killifish species, whereas transferring SW-acclimated fish to FW significantly decreases their expression (McCormick et al., 1989; McCormick, 2001; Marshall et al., 2002). To cope with dehydration caused by water loss in SW, similar to other euryhaline species, *A. fasciatus* must drink water and excrete excess NaCl by actively transporting Na^+ through the basolateral Na^+/K^+-ATPase, coupled with the secondary active transport of Cl^- via the NKCC, which then exits the cell through the apical CFTR channel (Marshall et al., 2002). For both transporters (ATPase and NKCC), the response to high-salinity conditions in terms of protein or transport activity was only marginally reflected by their respective transcript

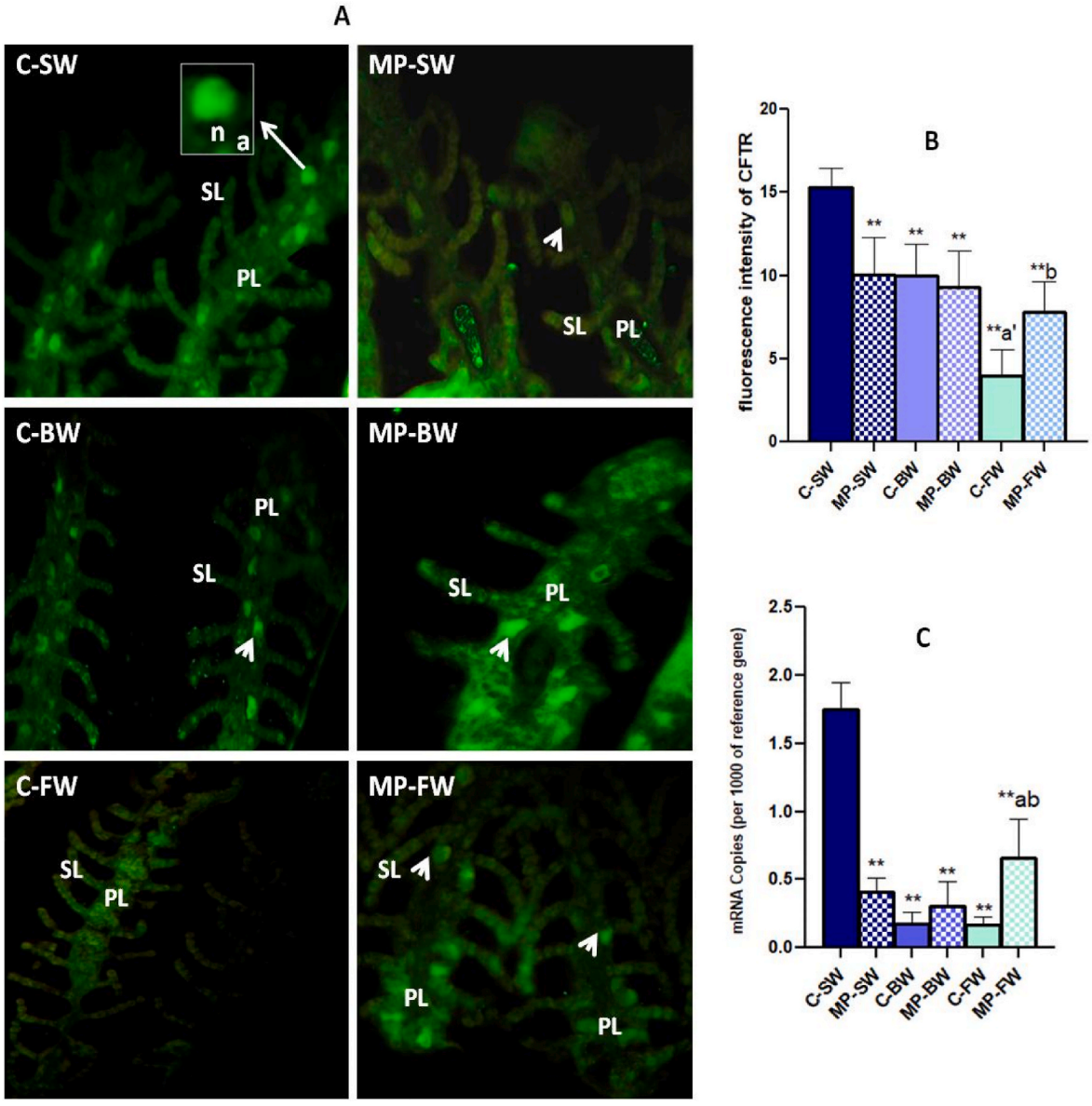

Fig. 8. Immunolocalization (**A**), fluorescence intensity (**B**), and gene expression (**C**) of the cystic fibrosis transmembrane regulator in the gills of fish acclimated to **SW** and those transferred to **BW** and **FW** in the absence (**C-SW, C-BW, and C-FW**) or presence of polystyrene microplastics (**MP-SW**, **MP-BW,** and **MP-FW**).
SL: secondary lamellae; PL: primary lamellae; n: nuclei of ionocytes; arrow head: ionocytes; arrows: apical fluorescence of CFTR in ionocyte; **: 0.01 versus SW; a: $p < 0.05$ and a': $p < 0.01$ versus BW; b: $p < 0.05$ versus FW(Gr:×40, insets "a": x 100).

levels. A similar situation has been observed in response to high-salinity in other fish, and has been attributed to various post-transcriptional regulatory mechanisms operating during high-salinity acclimation (MacKenzie et al., 2002; Shwe et al., 2020). During FW, fish experienced passive water gain and diffusive ion loss. Data regarding killifish ionocyte changes in FW are scarce; however, evidence suggests that these cells increase in size while maintaining basolateral Na^+/K^+-ATPase and apical NKCC expression (Katoh et al., 2003, 2008), which was confirmed to some extent in this study. Our results clearly indicated that a decrease in water salinity was associated with a significant decrease in the expression of the three transporters studied, with the lowest values consistently recorded in FW. The toxicity of PS-MPs was primarily manifested in FW by a decrease in Na^+/K^+ activity and the expression of the corresponding gene, as well as by a significant ($p < 0.01$) and unexpected increase in the tissue and gene expression of CFTR. Xue et al. (2022) reported a 60% reduction in gill Na^+/K^+-ATPase activity in zebrafish exposed to artificially degraded polyethylene MPs after five days in freshwater. Such modifications in ionocyte transporters could potentially disrupt osmoregulation during FW by decreasing the active basolateral uptake of Na^+ and promoting apical loss of Cl^-ions. Indeed, several studies have indicated that certain isoforms of Na^+/K^+-ATPase, such as NKA α1a (Chandrasekar et al., 2014), are expressed under hypo-osmotic conditions and are involved in the active uptake of Na^+ and Cl^-. However, gill CFTR expression should be undetectable in fish acclimated to FW, and Cl − excretion through the apical CFTR of the gill epithelium is essential only for seawater acclimation (Ouattara et al., 2009; Evans et al., 2005). Several authors have noted the disappearance of apical CFTR within a few hours of its transfer from SW to FW (Marshall et al., 2002; Scott et al., 2005; Tang and Lee, 2011). Therefore, the persistence of relatively high CFTR expression after 15 days of acclimation of *A. fasciatus* to MP-FW was abnormal, indicating a toxic effect of PS-MP that may negatively impact osmoregulation.

The paracellular pathway is also crucial for regulating the permeability of the fish gill epithelium (Evans et al., 2005). In SW, the gill

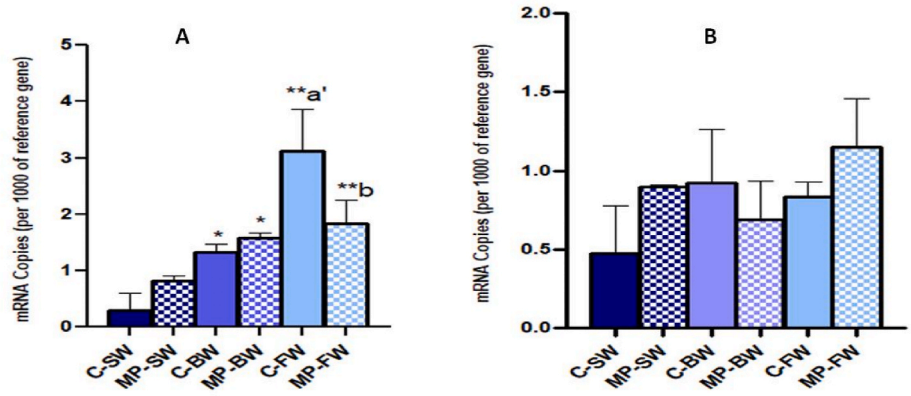

Fig. 9. Gene expression of claudin 3 (**A**) and claudin 4 (**B**) in gills of fish acclimated to **W** and those transferred to **BW** and **FW** in the absence (**C-SW, C-BW, and C-FW**) or presence of microplastics (**MP-SW, MP-BW, and MP-FW**).
*: $p < 0.05$ and **: $p < 0.01$ versus SW; a': $p < 0.01$ versus BW; b: $p < 0.05$ versus FW.

epithelium is "leaky," and paracellular permeability is important for facilitating Na^+ excretion, whereas in FW, the epithelium becomes "tight" to prevent passive ion loss (Chasiotis et al., 2008). The paracellular pathway is regulated by the TJ complex between epithelial cells, which is composed of transmembrane and peripheral scaffolding proteins (Bagherie-Lachidan et al., 2008). Claudins are a large family of integral transmembrane TJ proteins and are major determinants of paracellular permeability (Van Itallie and Anderson, 2006). Changes in the mRNA levels of certain claudin isoforms have been observed in the gills of euryhaline fish in response to variations in salinity (Tipsmark et al., 2008; Duffy et al., 2011). Bagherie-Lachidan et al. (2008) found that a decreased abundance of Cldn 3 mRNA in euryhaline fish, *Tetraodon biocellatus*, occurs in "leakier" epithelia and suggested that Cldn 3 TJ proteins likely play an important role in maintaining osmoregulation in euryhaline species. Accordingly, by evaluating the number of Cldn 3 and Cldn 4 mRNA copies in the gills of fish from different groups, we observed that Cldn 4 was insensitive to variations in water salinity, where as Cldn 3 increased with decreasing salinity. However, the significant decrease in Cldn 3 mRNA copy number noted in fish from the MP-FW group reflects the toxic effect of MPs, which may be responsible for maintaining a certain permeability of the gill epithelium in FW and, consequently, passive ion loss.

In conclusion, our study revealed that 15 days of exposure of the euryhaline fish *A. fasciatus* to environmentally relevant concentrations of PS-MPs altered osmoregulation, particularly in FW, indirectly through their toxic effects on gill tissue, directly by maintaining relatively high paracellular permeability through the reduction in the expression of Cldn 3, and by modifying the expression of transcellular transporters Na^+/K^+-ATPase and CFTR in the gill epithelium. Although the present study yielded important results concerning the direct effects of PS-MPs on the permeability of the gill epithelium in response to reduced water salinity, more comprehensive studies are required to investigate the mechanisms underlying the translocation of PS-MPs into the gill tissue and their toxicity. It is also important to note that *in vivo* studies and laboratory conditions do not fully reflect field conditions, where salinity fluctuations can occur daily and where gills are in contact with MPs of varying sizes, shapes, and polymers. Thus, future *in situ* studies should focus on the effects of environmental MPs on the osmoregulation of euryhaline fish. Overall, these results confirm the toxic effects of PS-MPs on the gill tissue and demonstrate for the first time that environmentally relevant concentrations of MPs adversely affect the permeability of the gill epithelium during decreased salinity acclimation in the euryhaline fish *A. fasciatus*.

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CRediT authorship contribution statement

Amira Abbassi: Methodology, Investigation, Data curation. **Kaouthar Kessabi:** Methodology, Investigation, Data curation. **Marta Casado:** Software, Investigation, Formal analysis. **Amalia Pérez-Jiménez:** Visualization, Investigation, Data curation. **Cristina E. Trenzado:** Supervision, Investigation, Data curation. **Eva E. Rufino-Palomares:** Writing – review & editing, Visualization, Data curation. **Hamadi Guerbej:** Validation, Resources, Methodology, Investigation. **Benjamin Piña:** Writing – review & editing, Visualization, Funding acquisition, Conceptualization. **Imed Messaoudi:** Writing – original draft, Visualization, Supervision, Methodology, 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

Data will be made available on request.

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