Comparative Biochemistry and Physiology, Part A 295 (2024) 111669

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

The effects of microplastics on ionoregulatory processes in the gills of freshwater fish and invertebrates: A prospective review

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ARTICLE INFO

Edited by Michael Hedrick

Keywords:
Functional groups
Organic matter homologues
Ion flux
Membrane disruption
Reporting requirements

ABSTRACT

From review of the very few topical studies to date, we conclude that while effects are variable, microplastics can induce direct ionoregulatory disturbances in freshwater fish and invertebrates. However, the intensity depends on microplastic type, size, concentration, and exposure regime. More numerous are studies where indirect inferences about possible ionoregulatory effects can be drawn; these indicate increased mucus production, altered breathing, histopathological effects on gill structure, oxidative stress, and alterations in molecular pathways. All of these could have negative effects on ionoregulatory homeostasis. However, previous research has suffered from a lack of standardized reporting of microplastic characteristics and exposure conditions. Often overlooked is the fact that microplastics are dynamic contaminants, changing over time through degradation and fragmentation and subsequently exhibiting altered surface chemistry, notably an increased presence and diversity of functional groups. The same functional groups characterized on microplastics are also present in dissolved organic matter, often termed dissolved organic carbon (DOC), a class of substances for which we have a far greater understanding of their ionoregulatory actions. We highlight instances in which the effects of microplastic exposure resemble those of DOC exposure. We propose that in future microplastic investigations, in vivo techniques that have proven useful in understanding the ionoregulatory effects of DOC should be used including measurements of transepithelial potential, net and unidirectional radio-isotopic ion flux rates, and concentration kinetic analyses of uptake transport. More sophisticated in vitro approaches using cultured gill epithelia, Ussing chamber experiments on gill surrogate membranes, and scanning ion selective electrode techniques (SIET) may also prove useful. Finally, in future studies we advocate for minimum reporting requirements of microplastic properties and experimental conditions to help advance this important emerging field.

1. Introduction

Microplastics are now ubiquitous in aquatic environments globally. They are defined as plastic polymers that range from 1 μm – 5 mm. Primary microplastics are manufactured in this size range; secondary microplastics are degraded to this size range from larger plastic products (Sun et al., 2020). There are numerous types of plastics, such as polyethylene, polystyrene, and polyvinyl chloride, that when coupled with the myriad of potential additives at manufacturing make "plastics" an umbrella term for an innumerable group of polymers that vary in structure. In recent years, there have been a number of projects launched to standardize the detection and quantification of plastics with focusses on increased accessibility (Munno et al., 2020) and customizable methods (Lei et al., 2022). Shi et al. (2024) recently concluded that degradation processes may change the nature of functional groups on the exposed surfaces of microplastics.

Physiological research on microplastic effects is a newly emerging field. Microplastics interact with aquatic organisms externally through adherence or entanglement, and internally through ingestion. The relative proportion of waterborne versus foodborne microplastic exposure has not yet been deduced and there is considerable debate whether microplastics cause pathological effects additional to those posed by any other type of particulate matter (i.e., digestive or respiratory blockage, increased energy allocated to the clearing/filtering of particles) (Zhu et al., 2023). The lack of standardization in experimental design, quantification (i.e. expression of concentration), and minimum reporting requirements (i.e. size, shape, and composition of microplastics) are primary drivers of this uncertainty. Additionally, we believe that microplastics are dynamic in that they change over time as they degrade, and this is rarely considered. All currently available knowledge should

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https://doi.org/10.1016/j.cbpa.2024.111669
Received 5 April 2024; Received in revised form 24 May 2024; Accepted 24 May 2024
Available online 26 May 2024

be interpreted with caution, in light of these uncertainties.

In freshwater animals, the gill is the primary organ of ionoregulation, especially for Na^+, Ca^{2+}, and Cl^-. The complex branchial structure has been reviewed by Wood and Eom (2021) in the context of the osmo-respiratory compromise. This is the trade-off between maintaining conditions in the gill that optimize respiratory gas exchange versus maintaining conditions that optimize the regulation of ion and fluid homeostasis. The gill serves as: (1) a physical barrier between internal and external environments limiting ion and nutrient loss, water entry, and foreign body penetration; (2) a differentially permeable membrane to maintain electrical gradients; (3) a site for active ion uptake and acid-base exchange mechanisms; and (4) an expansive functional surface area for gas exchange and waste excretion (Evans, 1987). All of these functions play into the osmorespiratory compromise and thereby impact ionoregulatory homeostasis at the gills; microplastics could potentially disrupt all of them (Griffith, 2017). Additionally, the gut, as a route of ion uptake from the food and the kidney (or its analogous organ in invertebrates), which is tasked with water excretion and ion conservation, are additional potential targets, but beyond the scope of this review.

At present, we do not understand the exact mechanism(s) by which microplastics are toxic to ionoregulation, and the goals of this prospective review are to summarize the current evidence and propose the paths forward to reach this understanding. First, we review the very few studies that report directly on the effects of microplastics on branchial ionoregulation in freshwater organisms. However, there are a number of others, also reviewed here, where indirect inferences about ionoregulatory effects can be drawn. Here we distinguish direct effects as those where a change in iono/osmoregulatory status was documented, from indirect effects as those where a change of iono/osmoregulatory status may have occurred based on other evidence but was not documented. Furthermore, we develop the thesis that there may be instructive analogies between the effects of microplastics and the effects of dissolved organic matter (commonly called dissolved organic carbon or DOC), where much more is currently known. Although DOCs (operationally defined as <0.45 μm diameter by most workers; Thurman, 1985) are smaller than microplastics (> 1 μm), both are carbon-rich, carry charge, share many of the same functional groups, and are subject to the same degradation processes in the environment as detailed below. Finally, we propose future research directions to illuminate the possible ionoregulatory effects of microplastics at a mechanistic level, and reporting guidelines that emphasize the importance of rigorous characterization of the tested materials.

1.1. Currently known direct effects of microplastics on branchial ionoregulation

We are aware of only four studies (one actually in brackish water) assessing direct effects on ionoregulation, and both methods and findings were variable. Watts et al. (2016) reported that green shore crabs (*Carcinus maenas*) exposed for 24 h to polystyrene microspheres (8 μm, 10^7/L, with or without carboxylated or aminated surface coatings), in full strength sea water (a salinity where they osmoconform) exhibited small, transient reductions in hemolymph [Na^+] and increases of hemolymph [Ca^{2+}]. However, in brackish water (10 ppt, where the crabs osmoregulate), there were no effects. Similarly, goldfish (*Carassius auratus*) exposed in fresh water to virgin polyvinyl chloride fragments (0.1–1000 μm, though <10% were >310 μm, 0.1–0.5 mg/L) for 4 days exhibited little or no effects on plasma ion levels, antioxidants, or Na^+, K^+-ATPase and H^+-ATPase activities in gills (Romano et al., 2020). On the other hand, Xue et al. (2022) noted consistent 60% reductions in gill Na^+,K^+-ATPase activity in zebrafish (*Danio rerio*) exposed to three size classes (45–53, 90–106, 250–300 μm) of artificially degraded polyethylene microspheres (all at 10^4/L) for 5 days in fresh water. Finally, Zheng and Wang (2023) exposed medaka (*Oryzias melastigma*) to polystyrene microbeads (0.4, 4, and 20 μm, all at 0.2 mg/L, and of proprietary composition) for up to 24 h in fresh water. Transient disturbances in Na^+, K^+ and Cl^- net flux rates and histopathological evidence of gill ionocyte damage were observed, effects which were both particle size-dependent and time-dependent. We conclude that microplastics can induce direct ionoregulatory disturbance, but the intensity depends on microplastic type, size, concentration, and exposure regime.

1.2. Observed indirect effects of primary microplastic exposure at the gill that may be associated with potential ionoregulatory consequences

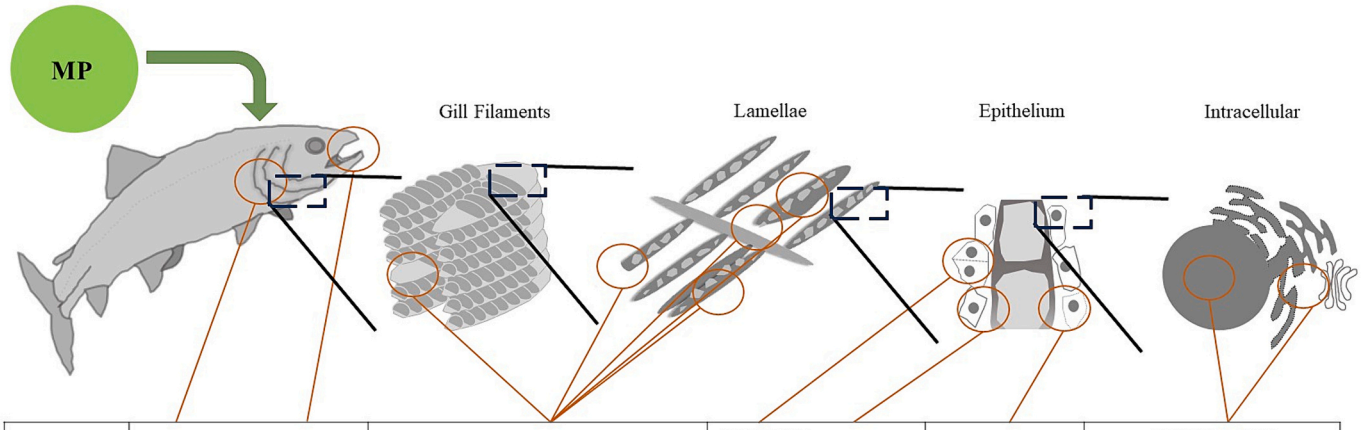
Fig. 1 outlines the findings of various studies across levels of biological organization and the potential correlative effects on ionoregulation. Organismal observations include a qualitative increase in mucus secretion, hypothesized to be a response to irritation of microplastics adhering to gill tissue (Limonta et al., 2019; Zeng et al., 2023; Zheng and Wang, 2023). Mucus provides a protective barrier between ambient water and gill tissue, which helps to stabilize ionic gradients (Shephard, 1992). Further, mucus likely plays an important role in CO_2 excretion, acid-base regulation and ammonia excretion as it is rich in carbonic anhydrase (Handy and Eddy, 1991). The ventilatory rate of fish first increased and then decreased under prolonged exposure to microplastics (Xue et al., 2021, 2022). Both altered breathing and increased mucus production could affect respiratory gas exchange and diffusive ions losses by the osmo-respiratory compromise (Wood and Eom, 2021). Histological evidence of gill damage by microplastics includes sloughing, fusion, hyperplasia, desquamation, and clubbed tips of the secondary lamellae, as well as epithelial lifting and cell proliferation (Karami et al., 2016, 2017; Limonta et al., 2019; Raza et al., 2023; Wang et al., 2022; Xing et al., 2023). These effects would likely compromise the barrier function, permeability, and transport properties of the gills, leading to increased ion losses, disturbed electrochemical gradients. and reduced active uptake rates (Evans, 1987).

Fig. 1 also describes some key intracellular effects that could be associated with ionoregulatory disturbance. These observations include enrichment of molecular pathways for peroxisome proliferators-activated receptors, protein digestion and absorption, p53 signaling, FoxO signaling, and CKD4/6 (Xing et al., 2023; Xue et al., 2022). Oxidative and possible nitrosative stress following microplastic exposure has been indicated by alterations in superoxide dismutase, catalase, malondialdehyde, and NO dynamics (da Araújo et al., 2022; Wang et al., 2022; Xing et al., 2023; Xue et al., 2021); intermediate consequences could involve protein denaturation, lipid peroxidation, and DNA hydroxylation. The downstream result of both could be apoptosis, and therefore damage to ionoregulatory functions. The most likely cause of oxidative stress is hypoxia in the gill tissue and associated hypoxaemia in the blood perfusing the gills. Both resulting from the morphological damage and enhanced mucus production highlighted in Fig. 1. The mitochondria of O_2-starved tissues emit reactive oxygen species (ROS), resulting in the sequence of damaging events described above (Johannsson et al., 2018; McGarry et al., 2018).

1.3. Recognizing the resemblance of microplastic surface characteristics to those of dissolved organic matter

The degradation of microplastics in aquatic environments occurs by five processes (Fig. 2): (1) photodegradation, especially by ultraviolet light, can cause surface changes in as little as 24 h; (2) thermal degradation often results in instability of the polymer; (3) mechanical degradation results from physical abrasion; (4) chemical degradation involves mechanisms such as exposure to digestive juices following ingestion; (5) biological degradation occurs through the colonization of microorganisms on the plastic surface (Sun et al., 2020). These degradative mechanisms result in an increase in the presence of functional groups on the microplastic surface, particularly carboxyl, hydroxyl, sulfide, amine, and aliphatic groups (Sun et al., 2020).

These same functional group are present and well-studied in organic



Effects of MP on the gill	↑ mucus secretion	Δ respiratory rate	Sloughing, Clubbed tips, Desquamation, Fusion, and Hyperplasia	Proliferation of epithelial and MRCs	Lifting	↑ apoptosis	↑ oxidative stress ↑ GSH activity ↓ AchE
Corresponding effects observed studying exposure to DOC	Exposure of fish to fulvic acids increases lysozyme (a protein present in many mucosal secretions) production at the gill		Histological damages including hyperplasia and clubbed tips	Proliferation of MRCs is observed in the gill when exposed to humic acid	Exposure to OM may cause lifting	Exposure to concentrated humic substances can cause apoptosis	Exposure to humic substances results in ROS creation, lipid oxidation, and ROS defense enzymes Glutathione stores are exhausted in exposure to humic substances, glutathione S transferase activity increases Esterase activity is altered by OM due to surface-activity causing permeability changes
Ionoregulatory consequence	Mucus is an important component in carbon dioxide excretion and stabilizes concentration gradients	Change in respiration affects ionoregulation by the osmo-respiratory compromise	Lamellae and filaments serve as an interface for ion and gas exchange between the blood and the external medium, alterations to lamellar structure that decreases the functional surface area of the lamellae alters ionoregulatory capacity	Damage to the gill epithelium can result in a loss of ability to maintain ionic concentration gradients		Impaired immune function and cellular structural integrity can inhibit ionoregulatory capacity	Oxidative and potentially nitrosative stress can hinder ion movement

Fig. 1. The effects of microplastic exposure (depicted in the first row) have been observed across levels of biological organization (illustrated from left to right). Many of the same effects have been observed following exposure to dissolved organic matter, commonly termed dissolved organic carbon (DOC) (second row). Though not explicitly explored in the literature to date as it relates to microplastic exposure, the observed effects may have potential ionoregulatory consequences (third row). MP = microplastic; OM = organic matter; MRC = mitochondria-rich cell.

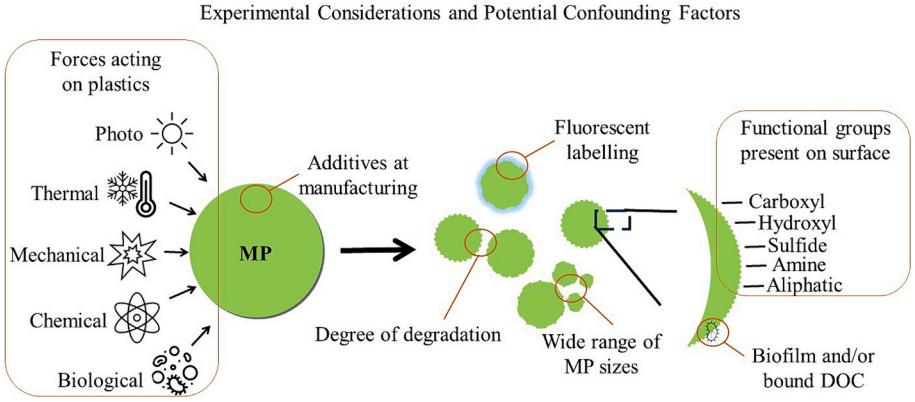


Fig. 2. The current lack of standardization of reporting microplastic characteristics makes the comparison and corroboration of studies difficult. The composition of plastics can be diversified at manufacturing through the introduction of fluorescent labels (particularly popular in microplastics produced for research) and additives. While in the environment, plastics are susceptible to a variety of forces resulting in photo, thermal, mechanical, chemical, and biological (resulting from biofilm production) degradation of the plastic surface. Over time, this creates plastics that vary in size and degree of surface degradation, including an increase in functional groups such as carboxyl, hydroxyl, sulfide, amine, and aliphatic groups on their surface.

matter. Carboxyl residues are abundant acidic groups which readily bind cations such as metals, many of which are known toxicants to fish gills(Galvez et al., 2008; Thurman, 1985; Wood et al., 2011). Sulfide groups, prominent in humic substances, also have a strong affinity for ions (Thurman, 1985). Amines are basic groups present in amino acids and some humic substances, which also exhibit strong affinities for ions (Thurman, 1985). Aliphatic groups are neutral and are also found in humic substances (Thurman, 1985). This combination of charged and uncharged groups provides hydrophilic and hydrophobic areas in the DOC molecules; these amphiphilic properties of DOC may promote its interaction with the gills (Wood et al., 2011). The same may be true of microplastics, and there is evidence that they become more hydrophilic as they weather, but remain overall hydrophobic (Mei et al., 2020), a change which would likely affect interaction with the gill.

It is well documented that various DOCs can either help or hinder ionoregulation in freshwater fish and crustaceans by altering active Na^+ uptake mechanisms, Na^+,K^+, ATPase activity, the transepithelial potential (TEP) across the gills, and their paracellular permeability (Duarte et al., 2016; Morris et al., 2021; Wood et al., 2011). While the mechanisms remain speculative, the greatest supportive or protective effects on ionoregulation are caused by freshly collected, optically dark DOCs of allochthonous origin which are rich in aromatic groups. Aromatic groups provide lipophilicity to parts of amphiphilic DOC molecules and may promote incorporation of part of the DOC molecule into gill cell membranes, even though other moieties of the molecule may carry strong negative charge (Morris et al., 2024). For example, functional groups on DOCs are thought to act on the paracellular junctions to differentially alter the permeability ratio of Na^+ to Cl^- (while decreasing absolute permeability), thereby making the TEP more negative (Galvez et al., 2008). Adsorption and/or absorption to the gills of neutral, lipophilic groups on the DOC may promote membrane integrity and stabilize permeability (Campbell et al., 1997; Vigneault et al., 2000). These supportive effects are attenuated or reversed to negative effects as DOCs progressively age, become smaller, lose aromatic rings, bleach, and become more lipophilic on an overall basis. Negative effects are also associated with optically lighter autochthonous DOCs with lower aromatic content. To our knowledge, there is no evidence for the presence of aromatic groups on the surfaces of microplastics themselves, and the apparent decrease in amphiphilicity and increase in hydrophilicity with weathering (Mei et al., 2020) may tend to decrease interactions with the gill surface.

On the other hand, the binding of natural DOCs to microplastics may increase the ability of the latter to interact with the gill surface. DOC coating of microplastics will likely increase with weathering time in the natural environment, and this may counteract the loss of amphiphilicity of the microplastics themselves with weathering described above. Aromatic, hydrophobic DOCs are the type that bind most readily to the surfaces of microplastics (Ding et al., 2020; Mei et al., 2020). Therefore, the presence of these surface-bound DOCs may in fact promote the ability of microplastics to exert effects on the ionoregulatory physiology of freshwater organisms in the real world.

To date, the very limited evidence available suggests that microplastics, like DOCs, may have either positive or negative effects on ionoregulation, as outlined in Fig. 1. Increased mucus secretion observed during exposure to both microplastics and fulvic acids has been attributed to a compensatory response to produce more lysozymes, a defensive protein present in mucosal secretions, with associated growth stimulation and improved immunocompetence and antioxidant defense in the case of fulvic acid (Lieke et al., 2021). In contrast, the histopathological changes, mentioned earlier (see above), caused by microplastic exposure may result in apoptosis and net ion losses across the gills, consequences also observed following exposure to high concentrations of some humic substances (da Costa et al., 2017; Sadauskas-Henrique et al., 2019, 2021; Steinberg et al., 2006). Increased oxidative stress following microplastic exposure may be synonymous to the oxidative stress observed in gills exposed to some humic substances, with notable increases in reactive oxygen species (ROS), lipid oxidation, and the upregulation of ROS defense enzymes (Steinberg et al., 2006). Increases in glutathione and decreases in acetylcholinesterase (AchE) activity induced by microplastics have also been seen with exposures to organic matter (Steinberg et al., 2006; Vigneault et al., 2000).

The correlative effects observed following exposure to microplastics versus DOCs suggest that functional group surface chemistry to be a common thread between these two classes of substances. The rich DOC literature provides both ideas and experimental approaches for probing the effects of microplastics on ionoregulation.

1.4. Future research directions

The following in vivo approaches that have greatly benefitted DOC research are recommended (Fig. 3). (**A**) TEP can be recorded using a high impedance voltmeter and Ag/AgCl electrodes in fish that have been fitted with an indwelling blood vessel or intraperitoneal catheter under anaesthesia, and then allowed to recover prior to measurements (Galvez et al., 2008; Sadauskas-Henrique et al., 2021). The graph in panel (**A**) shows our preliminary measurements of depolarization of TEP in goldfish acutely exposed to virgin polyethylene spheres (1–5 μm, 0.2 mg/L; L. Zink, C. Morris, C. M. Wood, unpubl.). (**B**) The measurement of net ion, ammonia, and acid-base flux rates between the fish and the water, as recently done for the first time in microplastics research by Zheng and Wang (2023) is a far more sensitive indicator of ionoregulatory disturbance and is less invasive than the measurement of plasma composition, as samples are taken only from the water phase in a closed system. This can be extended by the use of radiolabelled ions (e.g. $^{22}Na^+$) to separate unidirectional influx and efflux rates (Wood, 1992), and by the use of ^3H_2O (a transcellular flux marker) and ^{3}H-polyethylene glycol-4000 (a paracellular flux marker) to track water fluxes across the gills (Wood et al., 2019). (**C**) Concentration kinetic analyses of unidirectional influx rates of radiolabelled ions (e.g. $^{22}Na^+$, $^{45}Ca^{2+}$), provides valuable additional information on the Michaelis-Menten kinetics of the uptake transporters, which through Lineweaver-Burke type analyses yield estimates of the K_m (inverse of affinity) and J_{max} (maximum transport rate) (Matsuo et al., 2004; Wood, 1992). Increased K_m caused by microplastics would reflect competitive inhibition, whereas decreased J_{max} would indicate non-competitive inhibition (e.g. destruction of transport sites).

Fig. 4 illustrates more sophisticated in vitro approaches that could be used to pinpoint microplastic effects (**A**) Cultured gill epithelia on filters (Schnell et al., 2016) facilitate all the techniques outlined in Fig. 3 **A and B**, but in simplified preparations - for example, where MRCs are absent, so only effects on branchial diffusive permeability are seen (Galvez et al., 2008). (**B**) Ussing chambers provide these same benefits for flat epithelia such as the opercular membranes of killifish and tilapia that possess both pavement cells and abundant mitochondrial-rich cells (MRCs) and thereby serve as excellent gill surrogates (Mccormick, 1994). Ussing chambers simultaneously allow intermittent ion flux and continuous electrical measurements across the epithelia (e.g. TEP, conductance, short-circuit current). (**C**) The scanning ion-selective electrode technique (SIET; (Marshall and Bellamy, 2010) can also be used on opercular membranes and other gill surrogates to exactly pinpoint the cells through which ion fluxes are occurring, and to measure their intensity in the presence of foreign agents such as microplastics.

At present, there is virtually no information on the effects of microplastics and DOC on ionoregulation by the gills of seawater fish and invertebrates. All of the techniques outlined in **Figs. 3** and 4 have been used successfully in the past to characterize ionoregulation in marine animals and therefore hold promise in this regard. However one precautionary note is that those approaches using radioisotopes of major ions (Figs. 3B,C**, 4B**) require much greater absolute amounts of radioactivity and therefore expense, because of specific activity dilution by the much higher ion levels in seawater.

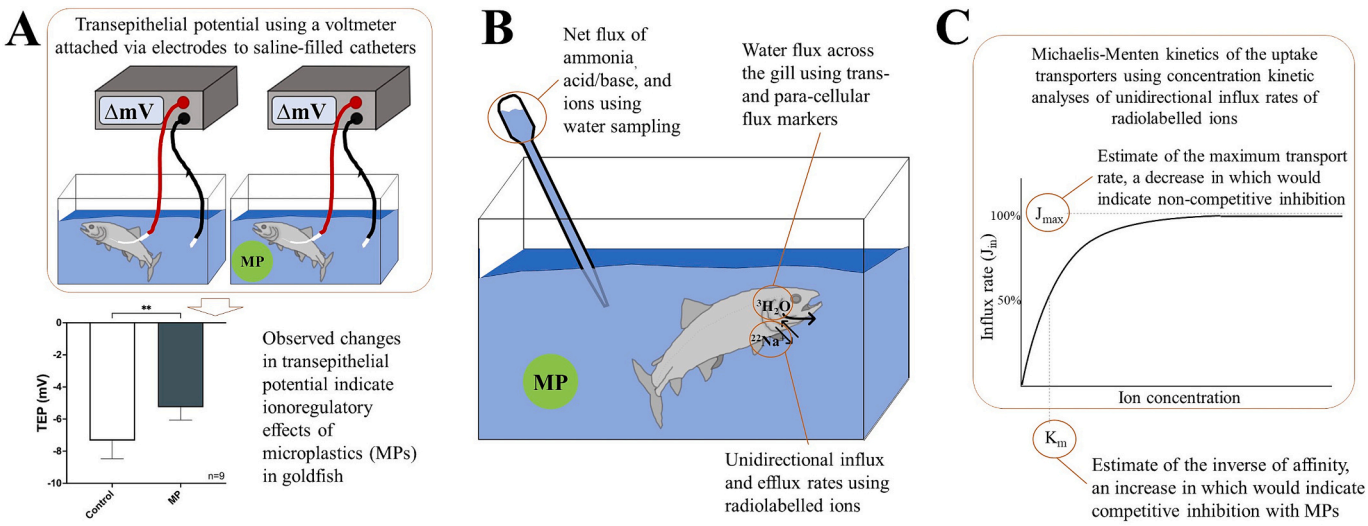


Fig. 3. Future research directions in vivo leveraging prior DOC research techniques include transepithelial potential (TEP) (**A**), ion fluxes and diffusive water exchange at the gills (**B**), and concentration kinetic analyses of unidirectional ion influx rates (**C**). MP = microplastic; ** indicates significant differences between groups, $p < 0.05$.

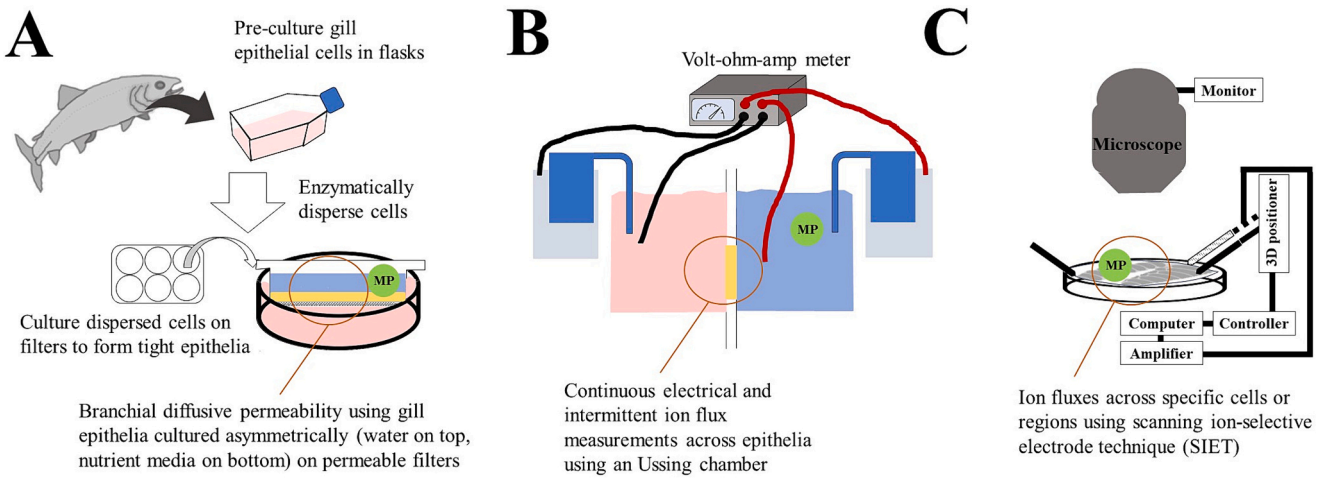


Fig. 4. Future research directions in vitro leveraging established methods such as culturing gill epithelia on filters (**A**), Ussing chamber (**B**), and scanning ion-selective electrode technique (**C**). MP = microplastic.

1.5. Critical experimental considerations

In conducting and reporting experiments, the importance of microplastic characterization cannot be overstated. Fig. 2 outlines some of the issues. Most microplastic studies to date have sourced plastic either from a manufacturer or through field collection, both of which may pose barriers in proper characterization – the former may be hindered by proprietary composition (including the introduction of substances to the surface, such as fluorescent labels), while the latter may include additives from the manufacturing process that are difficult to delineate, or have DOC and/or an established biofilm on their surface. Plastics that have undergone degradation will have altered surface characteristics, resulting in increased surface area, exposed functional groups, and widening size range due to fragmentation. A further complication is that in chronic exposures, plastics may degrade during the experiment. Therefore, adequate initial characterization as well as an understanding of how the experimental system may alter the plastics is imperative. To achieve this understanding, researchers should first establish the degradation rate of the microplastic of interest within the experimental system through time-course sampling regimes.

Table 1 outlines what we consider the minimum reporting requirements for future microplastic studies on ionoregulation. Careful consideration of these points should be taken so as to maximize information extractable from each study. Preliminary experiments will be helpful in meeting these goals. Firstly, microplastics should be well-characterized. As previously noted, barriers such as proprietary formulations and additives may make this challenging, but a surface characterization using techniques such as scanning electron microscopy and Fourier-transform infrared spectroscopy for functional group composition would provide insights on the plastic surface. Additional techniques are becoming well-substantiated with libraries of spectra including Raman spectroscopy, nuclear magnetic resonance spectroscopy, and near-infrared spectroscopy to compare polymer compositions. Shi et al. (2024) have recently published a comprehensive review on this topic, with a particular focus on changes that occur during weathering. To understand bioavailability, it is important to know particle size: large microplastics can impair physiological function through adhesion (such as gill clogging) while very small microplastics plastics may be able to infiltrate biological membranes. Over the course of the proposed experiment, the stability (state of degradation) of plastics and their concentration (which should be quantified in both mass/volume and particles/volume) should be monitored. As plastics can interact with

Table 1
Suggested minimum reporting requirements for studies assessing the effects of microplastics (MP) on ionoregulation.

Microplastic Properties	Quantification	Number of microplastics provided in in mass/volume and particles/volume
	Stability	Assessment of degradation of plastics in experimental setup over proposed experimental timeline
	Bioavailability	Method(s) used to keep MPs in suspension, or to incorporate MPs into food. Assessment of interaction (s) with the organisms resulting in interference to physiological functions (e.g., gill clogging, membrane destruction)
	Composition and Surface Characteristics	Shape, size, and composition of plastics. Functional group characterization of MP surface
Experimental Parameters	Water Quality	Composition of exposure water (including major ions, pH, DOC, water hardness, and alkalinity, temperature, O_2 and ammonia levels)
	Concentration Maintenance	Assessment of distribution in water column over time, determination of loss following water change or complexation with other molecules

ions and organic matter, these water quality characteristics should be reported.

CRediT authorship contribution statement

Lauren Zink: Writing – original draft, Investigation, Conceptualization. **Chris M. Wood:** Writing – original draft, Investigation, 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.

Acknowledgements

This work was supported by a Natural Sciences and Engineering Research Council of Canada Discovery Grant RGPIN-2023-03714 awarded to CMW and a Natural Sciences and Engineering Research Council of Canada Post-Doctoral Fellowship awarded to LZ. We thank two anonymous reviewers for their thoughtful and constructive comments that improved the paper.

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