Transepithelial potential remains indicative of major ion toxicity in rainbow trout (*Oncorhynchus mykiss*) after 4-day pre-exposure to major salts

Beverly H.K. Po, Chris M. Wood

*Department of Zoology, University of British Columbia, Vancouver, BC, Canada V6T 1Z4
Department of Biology, McMaster University, Hamilton, ON, Canada L8S 4K1

1. Introduction

Cases of prolonged salinization of natural rivers and lakes have been increasing in recent years, revealing the important consequences of unnaturally high concentrations of major ions for freshwater systems. These major ions (Na⁺, K⁺, Ca²⁺, Mg²⁺, Cl⁻, SO₄²⁻, and HCO₃⁻/CO₃²⁻) can have direct biological impacts, such as massive fish kills (Schulz and Canedo-Argüelles, 2019). To make it worse, more moderate contamination by salts can be accompanied by the freshwater salinization syndrome, a cascade of geochemical events that greatly reduce many aspects of water quality (Canedo-Argüelles, 2020; Kaushal et al., 2019).

It has been gradually established that the toxicity of the major ions is dependent on both the types of ion and the species in question, and that the cations seem to determine the toxicity more than do anions (Erickson et al., 2018, 2017; Mount et al., 2019, 2016, 1997). However, the environmental guidelines for major ions used by most governmental agencies have been largely based on toxicological studies which focused only on the toxicity of Cl⁻ and SO₄²⁻ and/or are expressed in nonspecific units such as total dissolved solids (TDS) or conductivity (e.g. CCME, 2011; Elphick et al., 2011a,b; USEPA, 1988, 2016). Therefore, the current regulatory tools are very crude, and there is growing agreement that future regulatory frameworks should incorporate ion-specific criteria for aquatic life (Canedo-Argüelles et al., 2019; 2016; Schuler et al., 2019; Soucek et al., 2011; Vander Laan et al., 2013). By traditional approaches, this would involve a great deal of classical toxicity testing, which is not only labor-intensive and expensive, but now increasingly difficult to perform in many jurisdictions, in light of animal welfare considerations.

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**ABSTRACT**

The Multi-Ion Toxicity (MIT) Model uses electrochemical theory to predict the transepithelial potential (TEP) across the gills as an index of major ion toxicity in freshwater animals. The goal is to determine environmental criteria that will be protective of aquatic organisms exposed to salt pollution. In recent studies, TEP disturbances above baseline (ΔTEP) during short-term exposures to major ions have been proven as indicative of their toxicity to fish, in accord with the MIT model. However, the acute 1-h exposures used in these previous studies might not be realistic relative to the 24 h or 96 h test periods used for toxicity assessment. To address this temporal inconsistency, the current study investigated both the TEP responses to serial concentrations of 10 major salts (NaCl, Na₂SO₄, NaHCO₃, KCl, K₂SO₄, KHCO₃, CaCl₂, CaSO₄, MgCl₂, MgSO₄) and plasma ion levels in juvenile rainbow trout after they had been pre-exposed to 50% of the 96h-LC50 levels of these same salts for 4 days. The pre-exposures caused no mortalities. In general, plasma ions (Na⁺, K⁺, Ca²⁺, Mg²⁺, Cl⁻) were well-regulated; however, pre-exposure to sulfate salts resulted in the greatest number of alterations in plasma ion levels. TEP responses remained largely similar to those of naïve trout (without salt pre-exposure). All salts caused hyperbolic concentration-dependent increases in TEP that were well-described by the Michaelis-Menten equation. In the pre-exposed trout, the variation of ΔTEP at the 96h-LC50 concentrations was only 2.2-fold, compared to nearly 28-fold variation among the molar concentrations of the various salts at the 96h-LC50s, identical to the conclusion for naïve trout. Overall, the results remove the temporal inconsistency of previous tests and remain supportive of the MIT model. In addition, the recorded alterations in certain plasma ions, baseline TEP, and Michaelis-Menten constants improve our knowledge on specific physiological responses after extended major ion exposure.
An alternate approach is modeling, based on existing toxicity data. The Multi-ion Toxicity (MIT) Model (EPRI, 2018, and previous versions cited therein) is one such approach. The MIT model attempts to predict major ion toxicity based on the assumption that disturbance of the calculated transepithelial potential (TEP) across the gills of aquatic animals is associated with the toxicity of ions, and that regardless of the mixture of ions causing the disturbance, there will be a constant TEP disturbance at the LC50 concentration. The model is based on the Spangler equation (Spangler, 1972), a generalized and somewhat complex extension of the Goldman-Hodgkin Katz equation (Goldman, 1943; Hodgkin and Katz, 1949; Pickard, 1976). It is used to predict the TEP resulting from exposures to mixtures of major ions in the external water. The advantage of the Spangler equation over the G-H-K equation is that it provides the flexibility needed to allow for the explicit inclusion of the concentrations of all of the major ions, rather than the three ions represented by the G-H-K form. In doing so, the model assumes that the internal ion levels in the organism stay constant, and allows the permeabilities of the organism’s epithelium to the various ions (which are key parameters in the equations) to vary, such that the permeabilities are actually “fitted” to the existing toxicity data. The lack of in vivo measured data of TEP in response to major ions has brought uncertainties in the application of the MIT model. However, in two recent studies (Po and Wood, 2021; Wood et al., 2020) on 5 species of teleost fish (fathead minnows, channel catfish, bluegills, rainbow trout, and goldfish) with 8–10 different salts, we have provided qualified support for the model. Key findings were that: (i) all salts tested depolarized the TEP (made it less negative) (ii) the individual salts varied greatly in their potency in depolarizing the TEP; (iii) the extent of the depolarization (ΔTEP) exhibited a hyperbolic response with respect to the concentration of each salt, reaching an asymptote close to the LC50; and (iv) most importantly, the ΔTEP values at the LC50 concentration were very uniform within a species, varying only 1.4 – 2.2 fold relative to 19–28 fold variation in the molar concentrations of the various salts at the LC50. Additionally, in two species (trout and goldfish), (v) changes in concentration of each salt, reaching an asymptote close to the LC50; and (vi) most (Po and Wood, 2021; Wood et al., 2020) on 5 species of teleost fish (fathead minnows, channel catfish, bluegills, rainbow trout, and goldfish) with 8–10 different salts, we have provided qualified support for the model. Key findings were that: (i) all salts tested depolarized the TEP (made it less negative) (ii) the individual salts varied greatly in their potency in depolarizing the TEP; (iii) the extent of the depolarization (ΔTEP) exhibited a hyperbolic response with respect to the concentration of each salt, reaching an asymptote close to the LC50; and (iv) most importantly, the ΔTEP values at the LC50 concentration were very uniform within a species, varying only 1.4 – 2.2 fold relative to 19–28 fold variation in the molar concentrations of the various salts at the LC50. Additionally, in two species (trout and goldfish), (v) changes in concentration of each salt, reaching an asymptote close to the LC50; and (vi) most.

While these results were encouraging, a key uncertainty lies in the time scale of the phenomena. For fish, the LC50 data were obtained from 24 h and 96 h exposures (the LC50 data of Mount et al., 1997 for fathead minnow), whereas the ΔTEP responses were measured immediately, after only a few minutes of exposure (Po and Wood, 2021; Wood et al., 2020). Indeed, the whole hyperbolic concentration-response relationships were recorded in less than 1 h, and the relative stability of plasma Na\(^+\) and K\(^-\) concentrations was measured after this 1 h of experimental exposures. Not only is there a temporal disconnect with the toxicity data, but also with the real world where elevated major ion concentrations may persist for extended periods.

Against this background, in the present study, we examined how prolonged exposure (96 h) to elevated concentrations of 10 different salts (at sublethal levels) affected the relationship between TEP and salt concentration in the rainbow trout. In separate experiments, we also assessed how these same sublethal exposures for 96 h affected a wider range of plasma ion concentrations (Na\(^+\), K\(^-\), Ca\(^2+\), Mg\(^2+\) and Cl\(^-\)). Based on the finding of Wood et al. (2020) that TEP responses were stable for 24 h in channel catfish (though only one concentration of each salt was tested), our working hypothesis was that concentration-response relationships in TEP and plasma ion levels would remain largely unchanged after these 96 h exposures in rainbow trout.

2. Materials and methods

2.1. Experimental animals

Experiments were performed under an approved UBC Animal Care Protocol (A18–0271). Juvenile rainbow trout (10 – 20 g) were obtained from the Freshwater Fisheries Society of British Columbia and were held at the University of British Columbia (Vancouver) for at least 6 months prior to experimentation in dechlorinated tap water at 10 – 12 °C. The size, origin, and holding conditions of the fish remained the same as in Po and Wood (2021). The composition of the Vancouver tap-water is (in mmol/L): Na\(^+\) = 0.063, Ca\(^2+\) = 0.075, Mg\(^2+\) = 0.007, K\(^+\) = 0.004, Cl\(^-\) = 0.050, SO\(_4^{2-}\) = 0.015; hardness = 10 mg CaCO\(_3\)/L, alkalinity = 8 mg CaCO\(_3\)/L and pH 7.0. This is a low-ion soft water. The fish were fed Nutra RC™ pellets (0.6 mm) at 1.5% ration three times a week until 1 day before the start of the exposure experiments.

2.2. Exposure solutions and animal holding

The exposure solutions were the single salt solutions of NaCl, Na\(_2\)SO\(_4\), NaHCO\(_3\), KCl, K\(_2\)SO\(_4\), KHCO\(_3\), CaCl\(_2\), CaSO\(_4\), MgCl\(_2\) (prepared from equimolar MgCl\(_2\)●6H\(_2\)O), and MgSO\(_4\) (Sigma-Aldrich, BDH, Fisher-Scientific, Anachemia, all >99% analytical grade). Most of the solutions were prepared to be 50% of the 96h-LC50 concentrations of each salt reported for Pimephales promelas (Mount et al., 1997). The percentage was chosen as it was assumed that at these levels the fish were very unlikely to be lethally affected. However, actual LC50 values were not reported for two salts by Mount et al. (1997) - i.e. for NaHCO\(_3\), the 96h-LC50 was reported as “< 10.1 mM”, and for KHCO\(_3\) the 96h-LC50 was “< 5.1 mM”. Therefore, for these two salts, conservative 25% concentrations of these solutions were used. For CaSO\(_4\), Mount et al. (1997) noted that the 96h-LC50 was above its solubility limit (i.e. no lethal effect) so these concentration (14.5 mM) was used.

It should be noted that there are very few major ion toxicity values for rainbow trout in the literature, but the few available are not greatly different from those for P. promelas (see Supplementary Information in Po and Wood, 2021). At present it is not possible to experimentally obtain LC50 data for fish under the vertebrate animal research ethics regulations of the Canada Council for Animal Care. Therefore, as in Po and Wood (2021), we have employed the LC50 values for P. promelas (Mount et al., 1997), which also were the main fish data used for the MIT model (EPRI, 2018).

Solutions were prepared 1 day before the start of exposure by dissolving the respective salt in dechlorinated tap water and making up to a bulk volume in a larger holding tank held in the same environmental chamber where the exposures were carried out. The CaSO\(_4\) solution was filtered to remove undissolved precipitates on the next day. The measured concentrations of cations in the exposure waters were representative of the nominal concentrations (Table 1A).

In the environmental chamber, the experimental temperature was 12 °C and day-light cycle was 14h:10 h. For the 96 h exposures to each salt, 7 – 9 rainbow trout were transferred into a glass aquarium with 40 – 50 L of the exposure water (5 – 7 L per fish) and held there unfed for 96 h before blood collection or TEP measurement. Several replicates were performed for each exposure so as to yield enough fish for separate blood sampling and TEP experiments. Procedural controls in the low-ion acclimation water were run with every set of exposures so their overall N number was higher (N = 21). The aquaria were bubbled with air, and water was replaced by 70% daily renewal with respective salted water.

Water conductivities and pH values for the various pre-exposure waters are reported in Table 1B, and those of each series of test solutions are reported in Supplementary Table S1. The conductivities of the salt solutions were measured with a WTW portable conductivity meter (ProfiLine Cond3310). There were minimal daily variations in the average conductivities in the exposure solutions during the 4-day periods (Table 1B). The pH values for the control, NaHCO\(_3\) and KHCO\(_3\) exposures were measured with a Fisher-Scientific Accumet AP84 portable meter and Oakton WD-35,801 electrode. The pH of the NaHCO\(_3\) and KHCO\(_3\) solutions were raised from 7.2 in control water to 7.9 and 8.0 respectively (Table 1B).
were individually transferred to a 120 mm Hg (4000 rpm, 1 min) and kept on ice before storage at −80 °C for later analysis. Plasma was immediately collected from the top layer after centrifugation of the blood (2000 rpm, 5 min). Plasma samples were diluted and standard solutions were prepared with ultrapure water (Millipore Milli-Q Integral 10, Molsheim, France) to the respective concentrations of the control sample. Lanthanum chloride solution (prepared by dissolving LaCl₃ in concentrated HCl) was added to reach 10 mg La/ml of the diluted samples for cation analyses in water samples. The concentrations of Na⁺, K⁺, and Mg²⁺ in plasma were measured by coulometric titration using a Radiometer CMT 10 chloride meter (Radiometer, Copenhagen, Denmark).

### 2.3. Plasma ion measurements

On the fourth day (96 h) of salt exposure, fish in the treatment tanks were individually transferred to a 12 °C bath of neutralized MS-222 (0.25 g L⁻¹) prepared using the corresponding salted water for anesthesia; control water was used for anaesthetization of the procedural controls. For each fish, blood (140 – 380 μL) was sampled by caudal puncture with a modified Hamilton syringe that had been previously anaesthetized with 50% of the reported highest limit of 96h-LC50 of the salt for each test, which were the same concentrations as in Po and Wood (2021) by taking reference to the LC50 levels reported for fathead minnows (Mount et al., 1997). Plasma samples were used for cation analyses in water samples. The concentrations of chloride in plasma samples were measured by coulometric titration using a Radiometer CMT 10 chloride meter (Radiometer, Copenhagen, Denmark).

### 2.4. Transepithelial potential (TEP) measurements

Fish surgery and measurement of TEP followed the procedures in Wood et al. (2020) and Po and Wood (2021). On the third day into the salt exposure period, fish were anesthetized in the MS-222 solution as mentioned above for cannulation surgery. The gills were irrigated with the MS-222 salt solution during the procedure. A short saline-filled polyethylene PE50 catheter (Clay Adams™, Becton Dickinson) was inserted into the peritoneal cavity and later secured by an outer PE160 sleeve and silk sutures. The catheter that was sealed with a pin. After a brief recovery in a separate tank, the fish were returned to the exposure salt solution in the original holding aquarium until the TEP measurements on day 4.

The TEP across the gills was measured as the inside voltage relative to the outside voltage as 0 mV (Potts, 1984). A pHM 82 pH meter (Radiometer, Copenhagen, which served as a high impedance voltmeter) was used to measure the voltages via Ag/AgCl electrodes (World Precision Instruments). These electrodes were PE160 tubing filled with 3 M KCl that were connected to the measurement points with salt-bridges made of PE90 filled with 3 M KCl-agar (5%). The reference electrode stayed in the external solution while the measurement electrode was connected to the saline-filled intraperitoneal catheter during measurement. Absolute TEP at a given moment was the voltage difference, averaged from three separate measurements, between the intra-peritoneal catheter and the exposure solution, each corrected by an intervening junction potential measurement.

For each TEP measurement, we allowed a 2–3-minute period of stabilization after the fish had been gently transferred to a small chamber containing about 200 ml of the test solution. The first measurement was performed in fresh exposure water (Table 1A). Next, the TEP was measured in dechlorinated tap water, which gave the baseline TEP in the ion-poor acclimation water. TEP measurements were compared to this baseline value to calculate the change of TEP (ΔTEP). Thereafter, experimental solutions comprised a geometric series of 8 concentrations of the salt for each test, which were the same concentrations as in Po and Wood (2021) by taking reference to the LC50 levels reported for fathead minnows (Mount et al., 1997). The measurements were conducted from the lowest concentration and moved up along the gradient. A total of 6 – 12 trout were used as replicates for each salt series and each fish was subjected to fresh solutions for the measurements.

### 2.5. Statistical analyses

Data have been expressed as means ± SEM (N). Analyses were performed in R Studio (R version 3.5.1). Data were firstly checked for normality and homogeneity of variances and one-way or two-way Analysis of Variance tests were performed for comparisons of the TEP and ΔTEP values at LC50 with Tukey’s post-hoc HSD test used to identify specific differences among groups. Student’s t-tests were performed for comparisons at the same exposure concentrations with the dataset from Po and Wood (2021) where identical measurements had been made on naive trout. Kruskal-Wallis (K-W) tests were used for the non-parametric data of plasma ions and baseline TEP, followed by Dunn’s test for pairwise comparisons with the control.

Following the same analysis as in Po and Wood (2021), means of ΔTEP as a function of salt exposure concentrations were fitted by non-linear regression to the Michaelis-Menten (M-M) model (Eq. (1)). The model is commonly employed in enzyme and transport kinetics, and it has been used for analyzing the response of TEP in all salt series earlier (Wood et al., 2020):

\[
\Delta \text{TEP} = \Delta \text{TEP}_{\text{max}} \times \frac{[\text{Salt}]}{K_m + [\text{Salt}]}
\]

where: ΔTEP = change in TEP (mV) relative to baseline TEP in background water

\[
\Delta \text{TEP}_{\text{max}} = \text{maximum change in TEP (mV) relative to baseline TEP (Salt)}_{\text{ext}} \text{ (external) salt concentration (mmol/L)}
\]

\[K_m = \text{affinity constant (mmol/L) representing the [Salt]_{\text{ext}} associated with 50% of } \Delta \text{TEP}_{\text{max}}\]
3. Results

There were no mortalities caused by the pre-exposures of each salt. This likely reflected the steepness of the concentration-response curves with respect to the major ion levels (< 8% mortality at 50% of LC50 levels) in the original toxicity tests that derived the LC50 values.

3.1. Changes in plasma ions after 4-day exposure

The plasma ion levels of the procedural controls (N = 21) held under the same conditions as the exposed fish, but in ion-poor Vancouver tap water were 148.6 ± 2.9 mM for Na\(^+\), 4.33 ± 0.15 mM for K\(^+\), 4.36 ± 0.08 mM for Ca\(^{2+}\), 1.15 ± 0.03 mM for Mg\(^{2+}\), and 135.7 ± 2.3 mM for Cl\(^-\) (Fig. 1).

In general, none of the 4-day salt exposure treatments caused a substantial change in the whole set of plasma ions though there were some significant individual alterations in one or two specific ions in some treatments (Fig. 1). Out of 50 comparisons (5 plasma ions x 10 salt treatments) there were only 6 significant differences from the control. These were elevations in the concentrations of Na\(^+\) (by 20% under KCl exposure), K\(^+\) (by 28% under CaSO\(_4\) exposure), Ca\(^{2+}\) (by 25% and 37% under KSO\(_4\) and MgSO\(_4\) exposures, respectively), and Mg\(^{2+}\) (by 28% under MgSO\(_4\) exposure). MgSO\(_4\) was the only salt for which 4-day exposure caused both of the divalent cations (Ca\(^{2+}\) and Mg\(^{2+}\)) to rise. The only significant decline was associated with exposure to Na\(_2\)SO\(_4\) which caused a 13.5% decrease in Cl\(^-\). Overall, these results show that the plasma ion levels remain largely unchanged from those of the control group after 4 days of individual salt exposure.

3.2. Baseline TEP

The baseline TEP values (Fig. 2A) in ion-poor tap water are important as they are used in calculation of ΔTEP values, including those at the 96 h LC50 salt concentrations (Fig. 2B). There was no change in baseline TEP in the procedural control: transferring fish from the tap water tank in which they were held for 4 days to the testing container of fresh tap water resulted in an absolute TEP of −22.5 ± 2.4 mV (N = 13), the same level as in the 81 naïve fish (−22.1 ± 0.7 mV reported in Po and Wood (2021) (Fig. 2A). Baseline TEP values (in tap water) of trout from the salt pre-exposure treatments (in most cases at one half of the LC50) also remained similar to this absolute level except for the CaSO\(_4\) and MgCl\(_2\) treatments, both of which exhibited significantly more negative values, down to −30.8 ± 1.1 mV and −34.6 ± 4.1 mV (both N = 6), respectively (Fig. 2A).

3.3. Responses of absolute TEP and ΔTEP to the salt series

The absolute TEP, and thus the ΔTEP, responded to the geometric series of increasing concentration of each individual salt by becoming progressively more positive until an asymptote to saturation was reached at higher concentration. These relationships were typical of those seen previously in naïve trout (Po and Wood, 2021) (Fig. 3). All of the plots approached saturation in TEP (and ΔTEP) at salt concentrations close to the 24h- and 96h-LC50 values, which are reasonably similar. However, the two bicarbonate salt treatments were an exception. For both, the 96h-LC50 values are less than one third of the 24h-LC50 values, and for these salts, saturation occurred close to the 24h-LC50, and well above the 96h-LC50 concentration. For most of the salts, the maximum TEP at high salt concentrations was close to 0 mV. Supplementary Figs S1–S3 present the same information with the salt solutes TEP plots in pre-exposed fish as better separate the data at low concentrations.

In comparison to the patterns of the naïve fish, the trend of the absolute TEP plots in pre-exposed fish were generally very similar. However, there were some statistical differences in absolute TEP for some of the concentrations in the treatments of NaCl, NaHCO\(_3\), KHCO\(_3\), CaCl\(_2\), CaSO\(_4\) and MgCl\(_2\) (Figs. 3A1, 3C1, 4C1, 5A1, 5B1, 5C1; also in Supplementary Figs. S1–3). All of the significant shifts for absolute TEP were towards a more negative value than in the naïve fish. There were interactions between the lowest salt concentrations and exposure experience in the CaCl\(_2\) and MgCl\(_2\) series (2-way ANOVA, p < 0.05; Figs. 5A1, 5C1 and supplementary Fig. S3). Pre-exposure to these two salts tended to result in a more rapid increase of TEP towards 0 mV as the

![Fig. 1. Boxplot with data points showing the plasma ion concentrations (in mM ± S.E.) of rainbow trout after 4-day pre-exposure to individual major salts at non-lethal concentrations (50% of 96h-LC50, Table 1). Asterisk (*) indicates significant difference (K-W test and Dunn’s test, p < 0.05) between the pre-exposed group (N = 7 – 9) when compared with the control group (N = 21), in contrast to the hyphen (-) which shows no difference between them. Plots with different letters denote differences when comparing among all the groups (K-W test, p < 0.05).]
concentration of the respective salts increase. The response to CaCl$_2$ series showed a small decline after reaching a plateau, a phenomenon that was also observed in naïve rainbow trout.

The ΔTEP responses for the Na and K salts in pre-exposed fish were also similar to the naïve fish (Fig. 3 and 4). Significantly lower ΔTEP responses after pre-exposure were seen only for the KHCO$_3$ series; significantly higher ΔTEP responses occurred in the majority of the concentrations in the CaCl$_2$, CaSO$_4$, and MgCl$_2$ series (Figs. 5A2, 5B2, 5C2). The Michaelis-Menten model provided an excellent fit ($R^2 = 0.988–0.998$) for all data sets except for the MgSO$_4$ series ($R^2 = 0.971$) (Table 2A). Among the pre-exposure treatments in this experiment, the Michaelis-Menten constant $K_m$ values for NaCl, Na$_2$SO$_4$, and KHCO$_3$ were higher than for the other salts, ranging between 6.5–8.7 mM compared to 0.4–3.0 mM. The theoretical peak of ΔTEP ($\Delta$TEP$_{max}$) calculated from the Michaelis-Menten equation for most of the salt series ranged between 22.9–26.9 mV (in the NaCl, Na$_2$SO$_4$, NaHCO$_3$, K$_2$SO$_4$, KHCO$_3$, CaSO$_4$, and MgSO$_4$ treatments) and was highest in the MgCl$_2$ series (32.6 mV); intermediate levels of $\Delta$TEP$_{max}$ were seen in the KCl and CaCl$_2$ pre-exposure treatments. Compared with the naïve trout, there were significant increases in $K_m$ (i.e. decreased affinity) in the ΔTEP response to NaCl and Na$_2$SO$_4$ series after pre-exposure, while the $K_m$ decreased (i.e. affinity increased) as a result of pre-exposure to NaHCO$_3$ (Table 2A). For $\Delta$TEP$_{max}$, opposite trends were apparent between Na$_2$SO$_4$ and KHCO$_3$ versus NaHCO$_3$ – there was an increase in $\Delta$TEP$_{max}$ for NaHCO$_3$ but decreases for Na$_2$SO$_4$ and KHCO$_3$. In addition, all of the Ca$^{2+}$ and Mg$^{2+}$ salts pre-exposures resulted in higher $\Delta$TEP$_{max}$ values when compared with naïve trout. Overall, by 2-way ANOVA, the $K_m$ values from the two exposure experiences were not significantly different ($p = 0.089$), but $\Delta$TEP$_{max}$ values were different, and there were significant interactions between the factors of salts and exposure experiences (Table 2B).

The $\Delta$TEP values at the 96h-LC50 remained very consistent with those of the naïve fish for all Na$^+$ and K$^+$ salts, but they were significantly higher after pre-exposure to CaCl$_2$, CaSO$_4$, and MgCl$_2$ (Fig. 2B). Nevertheless, the variation of ΔTEP at the 96h-LC50 was only 2.2-fold (13.3 mV in NaHCO$_3$ vs 29.0 mV in CaCl$_2$), compared to nearly 28-fold among the 96h-LC50s of the salts, expressed as molar concentrations. Notably, the variation of ΔTEP at the 96h-LC50s from naïve fish was also 2.2-fold (12.1 mV in NaHCO$_3$ vs. 26.1 mV in Na$_2$SO$_4$).

4. Discussion

4.1. The similarity in TEP responses and plasma ions in pre-exposed fish relative to naïve fish

The transfer of the rainbow trout into elevated salt solutions (generally 50% of the LC50 concentrations) for 4 days before measuring the TEP responses in the respective salt series was designed to address the temporal uncertainties in our previous physiological assessments (see Introduction) of the MIT model (EPRI, 2018). Freshwater fish
markedly after 96 h pre-exposure, in accord with our original hypothesis, and in accord with the 24 h pre-exposure data of Wood et al. (2020). While there were some interesting quantitative differences, as discussed below, the most important finding was that the ΔTEP values at the 96 h-LC50 concentrations remained very consistent, varying only 2-2.2-fold, when compared to the 28-fold variation in the molar salt concentrations at the LC50s (Fig. 2B). This was exactly the same conclusion as for naïve trout; in fact, there were significant differences for only 3 of the 10 salts, and these differences were rather small. Similarly, there were relatively few significant changes (6 out of 50) in the plasma concentrations of five ions as a result of 96 h pre-exposure (Fig. 1), and again these were relatively small. Overall the present results provide further physiological support for the MIT model by removing previous temporal uncertainties.

4.2. Plasma ions remained largely unchanged after 4-day salt treatment

Blood plasma ion concentrations were extremely well regulated in the face of elevated water ion levels that approached one-third of plasma levels (for Na\(^+\) and Cl\(^-\)), approximately equalled plasma levels (for K\(^+\)), or exceeded plasma levels by up to 5-fold (for Ca\(^{2+}\)) and 8-fold (for Mg\(^{2+}\)) (cf Table 1. vs Fig. 1.). This reflects a remarkable ability of the rainbow trout for ionoregulatory homeostasis. Simulations with the Goldman-Hodgkin-Katz equation suggest that magnitudes of the few measured changes in internal concentrations would have had only small effects on TEP. This helps explain why TEP concentration-response relationships were not greatly altered by the various pre-exposures. However, several caveats should be noted. Firstly, the rainbow trout is a facultatively euryhaline species (though not normally at this small pre-smolt life stage), so other teleost species may not be as proficient at regulating these ions, all of which are much higher in sea water than in fresh water. Secondly, as only plasma ions were measured, this does not necessarily mean that intracellular ion levels were subject to similar homeostasis. However, disturbances in intracellular ions would have no direct effect on TEP at the gills (Potts, 1984). Finally, our protocol involved 5–6 days of fasting prior to sampling. In rainbow trout, Bermejo-Poza et al. (2019) reported that all of the plasma ions measured here remained stable after 5 days of fasting, except Cl\(^-\) which increased slightly. Regardless, our procedural controls had undergone the same period of fasting as our salt exposed fish, so our conclusions are not confounded by this factor.

Interestingly, with the exception of plasma [Mg\(^{2+}\)] elevation in fish exposed to MgSO\(_4\) (Fig. 1D), the plasma ions that did change were not the ones to which the fish were exposed. The decrease in plasma [Cl\(^-\)] could reflect an ability of SO\(_4^{2-}\) to substitute for Cl\(^-\) at the gill anion exchanger which provides the route for Cl\(^-\) uptake in freshwater fish, though this substitution not yet been proven (Griffith, 2017). SO\(_4^{2-}\) vs. Cl\(^-\) exchangers have been identified in the marine fish kidney (Takvam et al., 2021). Elevations in plasma [Ca\(^{2+}\)] in the face of K\(_2\)SO\(_4\) and MgSO\(_4\) exposure (Fig. 1C) suggests some interaction of SO\(_4^{2-}\) regulation with Ca\(^{2+}\) regulation, but the mechanism is obscure. However, the fact that the Cl\(^-\) salts of Ca\(^{2+}\) and Mg\(^{2+}\) did not lead to any alteration in plasma concentration, in contrast to the SO\(_4^{2-}\) salts, suggests that these two cations were less likely to be the causes of the alterations. Nevertheless, the fact that SO\(_4^{2-}\) exposure contributed to 5 of the 6 significant plasma ion changes suggests that SO\(_4^{2-}\) regulation requires more investigation (Griffith, 2017). Finally, the significant rise in plasma [Na\(^+\)] in fish exposed to sublethal KCl (Fig. 1A) probably reflects the intimate relationship between Na\(^+\) and K\(^+\) at the level of basolateral Na\(^+/K^+\)/ATPase. This enzyme energizes all ion transport at the gills and imports 2 K\(^+\) ions into the gill ionocytes in exchange for the export of 3 Na\(^+\) ions into the blood (Evens et al., 2005). If this transport is accelerated during KCl exposure to export K\(^+\) across the gills, thereby precluding plasma homeostasis of this very toxic cation (Fig. 1B), then plasma [Na\(^+\)] would rise. Additionally, a putative Na\(^+\) vs. K\(^+\) cation exchanger has recently been identified at the apical membranes of gill ionocytes in zebrafish (Clifford et al., 2022).

4.3. Unchanged baseline TEP and alterations in TEP responses after pre-exposure to Na\(^+\) and K\(^+\) salts

There were no changes in baseline TEP (Fig. 2A) as measured immediately after transfer from the 4-day pre-exposure solutions to ion-
poor Vancouver tap water, despite the significant increase in plasma $[\text{Na}^+]$ associated with the KCl treatment, and significant decrease in plasma $[\text{Cl}^-]$ associated with $\text{Na}_2\text{SO}_4$ treatment (Figs. 1A and 1E, respectively). The TEP in freshwater fish is considered a diffusion potential reflecting the greater permeability of the gill epithelium to cations (mainly $\text{Na}^+$) over anions (mainly $\text{Cl}^-$) (Eddy, 1975; Kerstetter et al., 1970; McWilliams and Potts, 1978; Potts, 1984; Potts et al., 1973). Therefore, this result suggests that these treatments did not affect these permeabilities, or at least their ratio. This is remarkable, in light of the fact that pre-exposure concentrations of $\text{Na}^+$ and $\text{Cl}^-$ were up to one-third of plasma concentrations (Table 1). However, it should be noted that the baseline TEP in ion-poor water will be driven by differential permeabilities in the efflux direction. As external ion concentrations are experimentally increased, TEP becomes less negative because
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Differential permeabilities in the influx direction become increasingly important in determining the TEP (Wood and Grosell, 2008), resulting in the hyperbolic concentration-response profiles (Po and Wood, 2021; Wood et al., 2020).

While there were no marked changes in these concentration-response profiles, there were some significant differences at specific concentrations, both in absolute TEP and ΔTEP (Figs. 3, 4). The nature of these changes was clarified by the Michaelis-Menten analysis (Table 2). After NaCl and Na₂SO₄ pre-treatments, higher Kₘ values occurred. Kₘ values are the affinity constants in the Michaelis-Menten model, such that a higher Kₘ indicates a lower affinity, and a lower Kₘ indicates a higher affinity. Therefore, the NaCl and Na₂SO₄ pre-treatments appear to have resulted in decreased differential cation vs anion permeability ratios in the influx direction, which could be interpreted as a homeostatic acclimation effect. Earlier, in an analysis across different salts and species, we have shown that Kₘ values are positively related to LC50 values (Wood et al., 2020). If this is also true within the same salt, these results suggest that after pre-exposure to sublethal levels of NaCl and Na₂SO₄, these salts become less toxic, which again would reflect a homeostatic acclimation response. The lower ΔTEP_{max} in the Na₂SO₄ pre-treatment (i.e. reduced depolarization at high external salt concentrations) also suggests an acclimation response. The opposite occurred with the NaHCO₃ pre-treatment where a lower Kₘ and a higher ΔTEP_{max} were seen (Table 2), suggesting a non-homeostatic sensitization response as a result of the NaHCO₃ pre-exposure. However, this did not occur with KHCO₃. As discussed by Po and Wood (2021), the TEP responses to bicarbonate salts are more complex than those to other salts, reflecting the additional influence of high external pH caused by the HCO₃⁻ anion. The HCO₃⁻ anion also appears to contribute to the greater toxicity of some bicarbonate salts (Harper et al., 2014; Hills et al., 2019). Finally, the lack of changes in Kₘ and ΔTEP_{max} values in the KCl and K₂SO₄ treatments (Table 2) suggests that neither sensitization nor acclimation occurred for these two salts. In future, toxicity testing would be needed to evaluate whether our predictions (acclimation vs. sensitization) based on changes in Michaelis-Menten constants are supported.
4.4. Alterations in baseline TEP and TEP responses after pre-exposure to Ca\(^{2+}\) and Mg\(^{2+}\) salts

The shifts towards more negative baseline TEPs by the divalent salt pre-treatments, which were significant for CaSO\(_4\) and MgCl\(_2\) (Fig. 2A), led to more pronounced increases in the ΔTEP responses (Fig. 5). Accordingly, the ΔTEP\(_{\text{max}}\) values from the Michaelis-Menten analyses in these pre-treatments were all significantly higher when compared with those of the naïve fish (Table 2). It is now well-established that the TEP of freshwater fish is very sensitive to environmental [Ca\(^{2+}\)], decreasing sharply when the animals are transferred to low background water [Ca\(^{2+}\)] (Fig. 2A), and increasing sharply with transfer to slightly higher background water [Ca\(^{2+}\)], as seen in Fig. 5 (Eddy, 1975; McWilliams and Potts, 1978; Po and Wood, 2021; Wood et al., 2020; Wood and Grossel, 2008). This is believed to be due to the ability of Ca\(^{2+}\) to reduce both the absolute permeability of the gill, as well as its differential permeability to cations vs. anions by titrating negative charges on the paracellular pathways (Cuthbert and Maetz, 1972; Hunn, 1985; McDonald and Rogano, 1986; Potts and Fleming, 1970). While these pathways become less permeable overall, their relative anion-to-cation selectivity increases, both of which will make the TEP more positive. The fact that baseline TEP decreased in ion-poor water after pre-exposure to high concentrations of calcium salts indicates that the ability of the gill to bind Ca\(^{2+}\) decreased as a result of the pre-exposures.

Based on the similarity of the results between Mg\(^{2+}\) and Ca\(^{2+}\) salts (Fig. 2A; Table 2), it is likely that the Mg\(^{2+}\) ion was causing similar effects on the gill pathways as Ca\(^{2+}\). Mg\(^{2+}\), as a component of water hardness, is often considered to have similar permeability-reducing properties as Ca\(^{2+}\), but overall, there is very limited knowledge on the handling of Mg\(^{2+}\) in the fish gill (Griffith, 2017). Notably, the high affinities for both Ca\(^{2+}\) and Mg\(^{2+}\), as represented by the very low K\(_{\text{m}}\) values, were maintained unchanged after the 4-day pre-treatments (Table 2). Thus, the effects of Ca\(^{2+}\) and Mg\(^{2+}\) pre-exposures on TEP were restricted to the baseline TEP, but not the TEP responses towards the salt series. Based on our previous discussion, we would predict that neither acclimation nor sensitization occurred, but again, toxicity testing would be required to test this prediction.

4.5. Conclusions

Our results have confirmed our general hypothesis that concentration-response relationships in TEP, as well as plasma ion levels, would remain largely unchanged after the 96 h pre-exposures to high but sublethal salt levels (representing half of the 96h-LC50 concentrations). These findings remove a temporal disconnect in our earlier validation (Po and Wood, 2021; Wood et al., 2020) of the MIT model (EPRI, 2018), such that now 96 h ΔTEP responses are being used to predict 96 h toxicity. This finding improves the ecological and management relevance of this modeling approach for predicting major ion toxicity, as long-term exposure is often the case in salinized freshwaters. The present data also improve our basic knowledge of ionoregulatory homeostasis and the electrophysiology of the gills in a model freshwater fish, the rainbow trout. The few exceptions, where specific changes did occur as a result of the 96 h pre-treatments, point to interesting areas for future physiological and toxicological investigation, particularly the indications of acclimation and sensitization in some of the altered Michaelis-Menten constants.

Author contributions

CMW and BHKP conceived the project. BHKP performed all experiments and analyzed the data. BHKP wrote the first draft of the paper and CMW edited it.

Declaration of Competing Interest

We declare no competing interests.

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Supplementary materials

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