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# Trans-epithelial potential (TEP) response as an indicator of major ion toxicity in rainbow trout and goldfish exposed to 10 different salts in ion-poor water☆

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ABSTRACT

Freshwater ecosystems are facing increasing contamination by major ions. The Multi-Ion Toxicity (MIT) model, a new tool for risk assessment and regulation, predicts major ion toxicity to aquatic organisms by relating it to a critical disturbance of the trans-epithelial potential (TEP) across the gills, as predicted by electrochemical theory. The model is based on unproven assumptions. We tested some of these by directly measuring the acute TEP responses to a geometric series of 10 different single salts (NaCl, $Na_2SO_4$, KCl, $K_2SO_4$, $CaCl_2$, $CaSO_4$, $MgCl_2$, $MgSO_4$, $NaHCO_3$, $KHCO_3$) in the euryhaline rainbow trout (*Oncorhynchus mykiss*) and the stenohaline goldfish (*Carassius auratus*) acclimated to very soft, ion-poor water (hardness 10 mg $CaCO_3$/L). Results were compared to 24-h and 96-h LC50 data from the literature, mainly from fathead minnow (*Pimephales promelas*). All salts caused concentration-dependent increases in TEP to less negative/more positive values, in patterns well-described by the Michaelis-Menten equation, or a modified version incorporating substrate inhibition. The ΔTEP above baseline became close to a maximum at the 96-h LC50, except for the $HCO_3^-$ salts. Furthermore, the range of ΔTEP values at the LC50 within one species was much more consistent (1.6- to 2.1-fold variation) than the molar concentrations of the different salts at the LC50 (19- to 25-fold variation). ΔTEP responses were related to cation rather than anion concentrations. Overall patterns were qualitatively similar between trout and goldfish, with some quantitative differences, and also in general accord with recently published data on three other species in harder water where ΔTEP responses were much smaller. Blood plasma $Na^+$ and $K^+$ concentrations were minimally affected by the exposures. The results are in accord with most but not all of the assumptions of the MIT model and support its further development as a predictive tool.



## 1. Introduction

Freshwater salinization, defined as an increase in concentration of major ions ($Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, $Cl^-$, $SO_4^{2-}$, and $HCO_3^-/CO_3^{2-}$), is now recognized as one of the top stressors on freshwater biodiversity (Cañedo-Argüelles et al., 2013; Cañedo Argüelles Iglesias, 2020; De Castro-Català et al., 2015; Vander Laan et al., 2013). However, as early as the 1940s in Spain and the 1960s in Germany, researchers have documented salinization (Schulz and Cañedo-Argüelles, 2019) and negative biological impacts including fish kills (Buhse, 1989; Illies, 1956; Schulz and Cañedo-Argüelles, 2019; Ziemann, 1967) downstream of potash mining sites. Other causes of freshwater salinization include mining for other resources (Palmer et al., 2010; Pond et al., 2008), agriculture (Allison et al., 1990; Halse et al., 2003), and road-deicing salts (Findlay and Kelly, 2011; Moore et al., 2020). Detrimental effects have been seen at organismal and population levels (Cañedo-Argüelles et al., 2013; Mount et al., 1997, 2016; Pond et al., 2008), in communities (Pond, 2010; Short et al., 1991), and in whole ecosystems (Berger et al., 2019; Hintz and Relyea, 2019; Schäfer et al., 2012). The situation is worsening (Cañedo-Argüelles et al., 2019; Kaushal et al., 2019). Human-induced weathering interacts with anthropogenic inputs of salts in a dynamic, synergistic way that can lead to diverse changes in

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water quality (salinity, pH, concentrations of base cations, nutrients and metals), creating the so-called freshwater salinization syndrome (Kaushal et al., 2019).

Earlier investigations focused on seawater intrusion and road salts. Since $Na^+$ (the most abundant seawater cation) is often considered relatively non-toxic, historically there has been more emphasis on the anions than on the cations. For this reason, the concentrations of the most abundant anions in the sea, i.e. $Cl^-$ and $SO_4^{2-}$, were often used for regulatory guidelines (CCME, 2011; USEPA, 1988). It is also recognized that hardness in test waters affects the toxicities of both anions (Elphick et al., 2011a, b). However, there is no general agreement on criteria used for regulation of major ion pollution (Schuler et al., 2019). Salinity, conductivity (measured as μS/cm), and total dissolved solids (TDS) have commonly been used (USEPA, 2016). All these are effective monitoring tools, especially conductivity because of its ease of continuous measurement. However, all of them assess complex mixtures of major ions simply by measuring a collective physicochemical property in the waterbody. Unless the chemical nature of salt contamination is known and relatively stable (e.g. road salt runoff) they are inadequate metrics for regulation because both the types of major ions and their various ratios are crucial in determining toxicity (Erickson et al., 2017; Hintz and Relyea, 2017; Mount et al., 2016; Schulz and Cañedo-Argüelles, 2019).

A consensus is now arising that future regulatory frameworks should incorporate ion-specific criteria for aquatic life (Canedo-Argüelles et al., 2016, 2019; Schuler et al., 2019; Soucek et al., 2011; Vander Laan et al., 2013). This will require extensive, standardized toxicity studies on all major salts, tested individually and in mixtures, and at acute and chronic concentrations. Currently, there are only limited examples (Erickson et al., 2017; Mount et al., 1997, 2016, 2019; Soucek et al., 2011). One way to reduce the experimental burden is to develop predictive models based on existing data (EPRI, 2018; Erickson et al., 2018; Mount et al., 2019).

The Electric Power Research Institute (EPRI) Multi-ion Toxicity (MIT) model (EPRI, 2018, and previous versions cited therein) is based on integrating predicted physiological responses with available toxicity data. The basic premises are (i) that changes in the concentrations of major ions will differentially disturb electrochemical gradients across biological epithelia (e.g. gills), resulting in disturbances in the transepithelial potential (TEP); and (ii) a certain disturbance of the TEP will be associated with incipient mortality. In the MIT model, the TEP is predicted by the Goldman-Hodgkin-Katz (GHK) Equation (Goldman, 1943; Hodgkin and Katz, 1949; Pickard, 1976):

$$TEP = \frac{RT}{F}\ln\left(\frac{p_K[K^+]_o + p_{Na}[Na^+]_o + p_{Cl}[Cl^-]_i}{p_K[K^+]_i + p_{Na}[Na^+]_i + p_{Cl}[Cl^-]_o}\right) \quad (1)$$

where:

$R$: universal gas constant (8.314 J/mol/K).

$T$: absolute temperature (K).

$F$: Faraday constant ($9.649 \times 10^4$ Coulombs/mole).

TEP: transepithelial potential (volts).

$p_{ion}$: permeability for subscripted ion (m/second).

$[ion]_o$: ion activity in the external water (mmol/L).

$[ion]_i$: ion activity in the extracellular fluid of the organism (mmol/L),

or by an extension (Spangler, 1972) that considers both divalent and monovalent ions. The MIT model is based on assumptions for which there was no experimental evidence at the time of model development. The model uses literature values for internal ion concentrations as approximations of true activities, and assumes that these remain constant during exposure. The permeabilities used in the model are also assumed values, based on the iterative fitting to toxicity data. This fitting allows changes in the relative permeabilities in different exposure solutions so as to best describe the data (i.e. auto-validation, rather than independent validation). Despite these many uncertainties, the MIT model has been used to describe major ion toxicity to several aquatic organisms, including both daphnids and fish, and showed promise with a freshwater mussel, but was unsuccessful with the mayfly *Neocloeon triangulifer* (EPRI, 2018). This exception might be related to the different ionoregulatory responses in the Ephemeroptera, where the stimulation of high ionic transport rates in high environmental $Na^+$ and $SO_4^{2-}$ exposures was suggested to significantly increase energetic costs of ionoregulatory homeostasis (Kefford, 2019; Scheibener et al., 2016; Buchwalter et al., 2019).

Recently, Wood et al. (2020) conducted the first experimental test of the MIT model, by making *in vivo* TEP measurements on fathead minnow (*Pimephales promelas,* FHM), channel catfish (*Ictalurus punctatus*, CC), and bluegills (*Lepomis macrochirus*, BG) acutely exposed to concentration series of eight different salts. The results provided support for the model as: (i) the disturbance in the TEP across the gills (ΔTEP) correlated well with the widely differing relative toxicities of the salts (96-h LC50 data for FHM from Mount et al., 1997), and (ii) the ΔTEP at the LC50 was very uniform. Both are in accord with the two basic premises of the model summarized above.

In the present study, our goal was to extend validation of the MIT model using two additional freshwater fish species, the rainbow trout (*Oncorhynchus mykiss*, RBT) and goldfish (*Carassius auratus,* GF). Both species have well characterized ionoregulatory mechanisms, and the electrical potentials across their gills have been studied (GF: Eddy, 1975; and RBT: Kerstetter et al., 1970; Kerstetter and Kirschner, 1972; Potts, 1984; Wood, 1991). The RBT is of additional interest because it is facultatively euryhaline. Toxicity data for major salts on these two species are fragmentary (Table S1), and it is now not possible to experimentally obtain LC50 data for fish under the vertebrate animal research ethics regulations of the Canada Council for Animal Care. Therefore the LC50 values for FHM (Mount et al., 1997), which was also the main fish database used for the MIT model (EPRI, 2018), have been employed. For those few salts where LC50 data were available for RBT and GF, they did not differ greatly from the FHM values (Table S1, Figs. 1 and 2). A second goal exploited the extremely soft, ion-poor nature of Vancouver water to see if the same principles applied as elucidated by Wood et al. (2020) in moderately hard Miami water (Table S2). $[Ca^{2+}]$, the major component of water hardness, has been variously reported to either reduce or increase major salt toxicity (Bogart et al., 2019; Davies and Hall, 2007; Elphick et al., 2011a, b; Erickson et al., 2017; Mount et al., 2016). A third goal was to examine responses to two additional salts ($NaHCO_3$, $KHCO_3$); bicarbonate salts, which are enriched in coal-mining effluent (Hills et al., 2019), are of special interest because they also raise water pH. A final objective was to measure $Na^+$ and $K^+$ concentrations in blood plasma after exposures to evaluate the MIT model assumption that these remain constant.

## 2. Materials and methods

### 2.1. Experimental animals

Experiments were performed under an approved UBC Animal Care Protocol (A18-0271). Juvenile rainbow trout (RBT, 10–20 g) were obtained from the Freshwater Fisheries Society of British Columbia and goldfish (GF, 3–11 g) were obtained from a local pet shop. Both species were held at the University of British Columbia (Vancouver) for 2+ weeks prior to experimentation in dechlorinated tap water at 10–12 °C for RBT, and 18 °C for GF. The

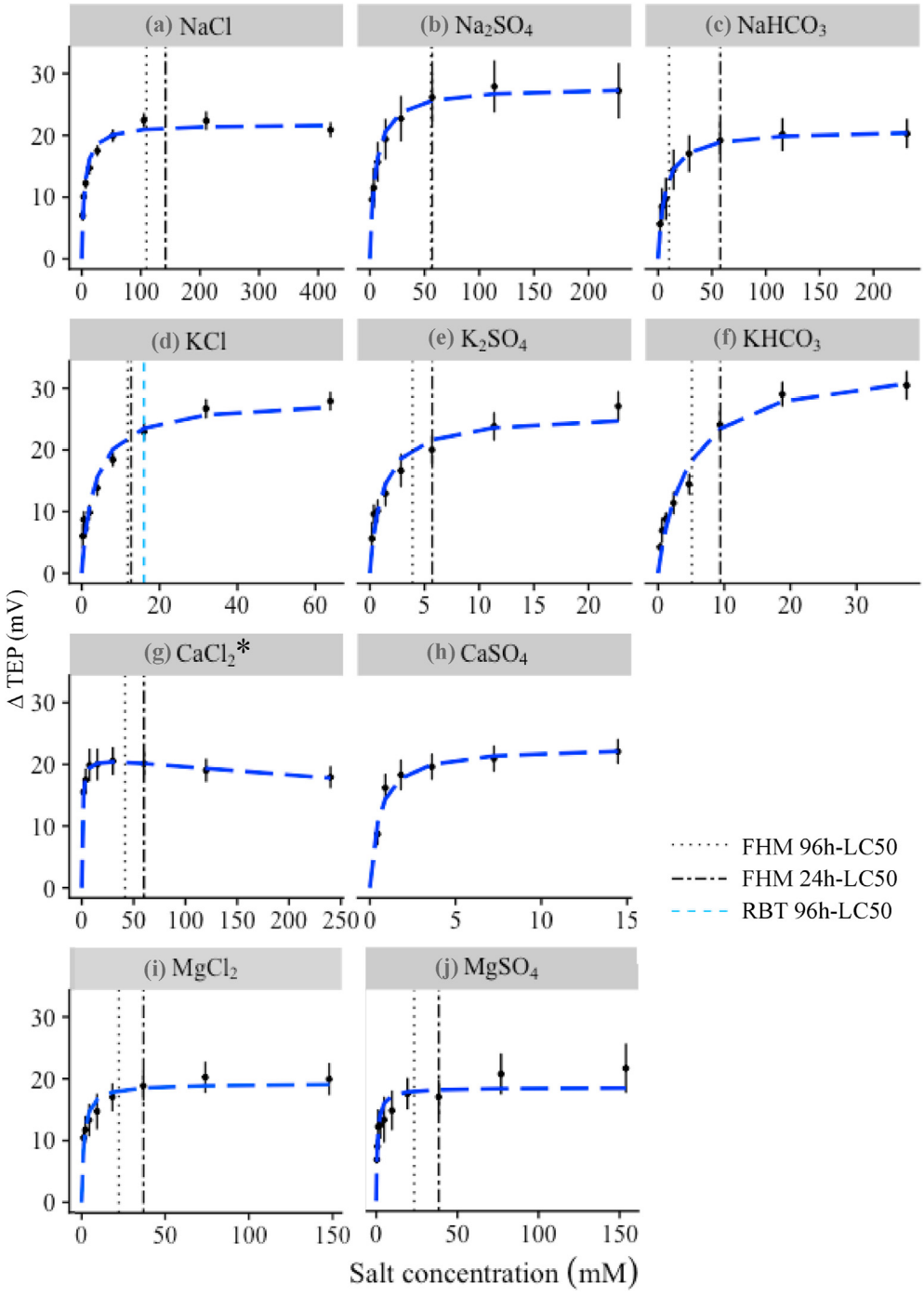


**Fig. 1.** Changes in transepithelial potential (ΔTEP) of rainbow trout (RBT) at different concentrations of major salts (mM) (Means ± SEM, N = 6–12), fitted by either the Michaelis-Menten model or by the model with substrate inhibition taken into account (marked by * next to salt name). Vertical lines indicate median lethal concentrations (LC50): 96 h-LC50 (dotted line) and 24 h-LC50 (dash dot line) reported for fathead minnow (FHM) in Mount et al. (1997), and 24h−LC50 for RBT (blue dashed line, from Durand-Hoffman (1995)). For $Na_2SO_4$, the 96h−LC50 and 24 h-LC50 points were less than 1 mM apart, so the lines are superimposed. For $CaSO_4$, the LC50 was above its dissolution concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

composition of the very soft 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. RBT were fed Nutra RC™ pellets (0.6 mm) and GF were fed Aquarian™ pellets at 1.5% ration every other day.

### 2.2. Experimental solutions

The ten single major salts tested were NaCl, $Na_2SO_4$, $NaHCO_3$, KCl, $K_2SO_4$, $KHCO_3$, $CaCl_2$, $CaSO_4$, $MgCl_2$ (prepared from equimolar $MgCl_2.6H_2O$), and $MgSO_4$ (Sigma-Aldrich, BDH, Fisher- Scientific,

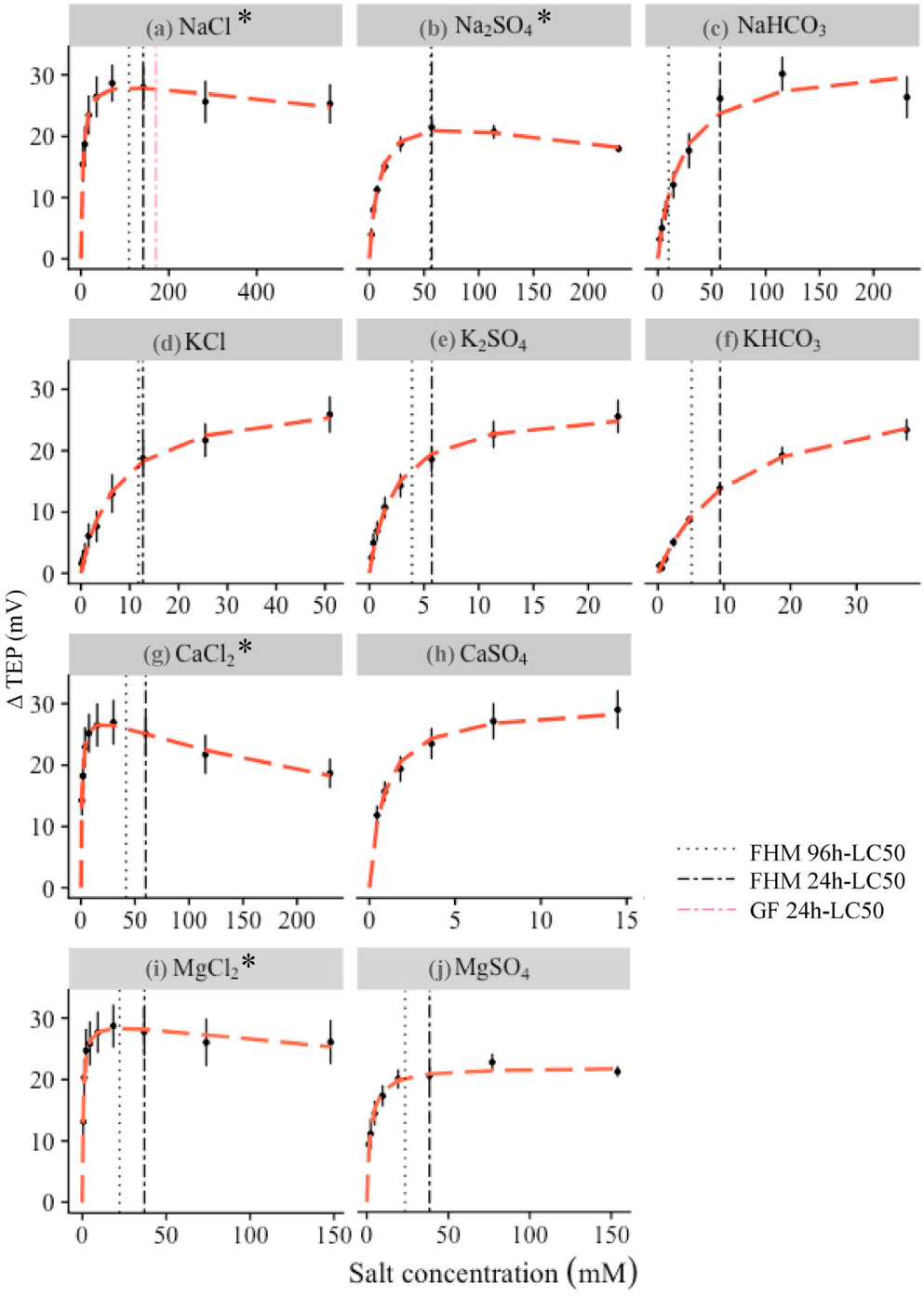


**Fig. 2.** Changes in transepithelial potential (ΔTEP) of goldfish (GF) at different concentrations of major salts (mM) (Mean ± SEM, N = 6–8), fitted by either the Michaelis-Menten model or by the model with substrate inhibition taken into the model (marked by * next to salt name). Vertical lines indicate median lethal concentrations (LC50): 96 h-LC50 (dotted line) and 24 h-LC50 (dash dot line) reported for fathead minnow (FHM) in Mount et al. (1997), and specific 24 h-LC50 for GF (pink dashed line, from Adelman and Smith, 1976). For $Na_2SO_4$, the 96h−LC50 and 24h−LC50 points were less than 1 mM apart, so the lines are superimposed. For $CaSO_4$, the LC50 was above its dissolution concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Anachemia, all >99% analytical grade). For each experiment, salts were freshly dissolved as concentrated stock solutions in de-chlorinated tap-water, then geometrical series were prepared by volumetrically diluting into de-chlorinated tap-water to obtain concentrations of 3.12%, 6.25%, 12.5%, 25%, 50%, 100%, 200%, and 400% of the 24 h-LC50 values (except the NaCl series in RBT, which used the 96 h-LC50 instead) derived mainly from toxicity data for FHM from Mount et al. (1997) and one literature value for trout (Table 1). $CaSO_4$ was an exception, because the LC50 value is above its maximum dissolution concentration, so the highest concentration chosen was close to the dissolution limit. Additionally, for NaCl in RBT and for $CaCl_2$ and $MgCl_2$ in GF, one more dilution (1.56%) was tested, to improve resolution at the lower end. The conductivities of all solutions were measured with a WTW portable conductivity meter (ProfiLine Cond3310), and the pHs for the bicarbonate salt solutions with a Fisher-Scientific Accumet AP84 portable meter and

**Table 1**
Michaelis-Menten constants (means ± SE) and $R^2$ values for the concentration-kinetics curves fitted empirically in Figs. 1 and 2, where the best-fit Michaelis-Menten model (higher goodness of fit) amongst the conventional (equation (2)) and the modified with substrate inhibition model (equation (3)) were plotted. All relationships were significant ($p < 0.05$) and the parameters from the best fit model are bolded. For both $\Delta TEP_{max}$ and $K_m$, A, B, C denote significant differences (Tukey HSD tests, $p < 0.05$) within a species among salts, whereas "^" denotes significant differences within a salt among species. Only the best-fit models were compared. For goldfish, all referenced concentrations of median lethal concentrations (Ref. LC50) for the test series were the 24 h-LC50 for fathead minnow (from Mount et al., 1997). For rainbow trout, most of them were the same 24 h-LC50 values except for the NaCl and KCl series (as described in the footnote).

| | Salt | Ref. LC50 (mM) | M-M Model | | | M-M Model with substrate inhibition | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | $\Delta TEP_{max}$ (mV) | $Km$ (mM) | $R^2$ | $\Delta TEP_{max}$ (mV) | $Km$ (mM) | $Ks$ (mM) | $R^2$ |
| **Rainbow trout** | NaCl | 109.3[a] | 21.9 ± 0.6$^{CD,\wedge}$ | 4.6 ± 0.7$^{AB}$ | 0.990 | NA | | | |
| | $Na_2SO_4$ | 56.9 | 27.9 ± 0.8$^{B}$ | 5 ± 0.6$^{AB,\wedge}$ | 0.993 | NA | | | |
| | $NaHCO_3$ | 57.7 | 20.9 ± 0.5$^{CD,\wedge}$ | 6.3 ± 0.7$^{A,\wedge}$ | 0.995 | NA | | | |
| | KCl | 16.0[b] | 28.2 ± 2.0$^{B}$ | 3.2 ± 0.9$^{BC,\wedge}$ | 0.977 | NA | | | |
| | $K_2SO_4$ | 5.7 | 25.9 ± 1.6$^{BC}$ | 1.1 ± 0.3$^{C,\wedge}$ | 0.978 | NA | | | |
| | $KHCO_3$ | 9.4 | 34.3 ± 2.3$^{A}$ | 4.4 ± 0.9$^{AB,\wedge}$ | 0.988 | NA | | | |
| | $CaCl_2$ | 60.0 | 19.8 ± 0.5 | 0.5 ± 0.2 | 0.989 | **21.4 ± 2.5$^{CD,\wedge}$** | **0.7 ± 0.1$^{C,\wedge}$** | **1191.4 ± 161.6** | **0.999** |
| | $CaSO_4$ | ≥14.5[c] | 22.9 ± 0.9$^{BCD,\wedge}$ | 0.5 ± 0.1$^{C}$ | 0.991 | NA | | | |
| | $MgCl_2$ | 37.0 | 19.3 ± 0.7$^{D,\wedge}$ | 1.5 ± 0.3$^{C,\wedge}$ | 0.981 | NA | | | |
| | $MgSO_4$ | 38.5 | 18.5 ± 1.0$^{D,\wedge}$ | 0.8 ± 0.2$^{C,\wedge}$ | 0.954 | NA | | | |
| **Goldfish** | NaCl | 141.7 | 27.6 ± 0.8 | 3.5 ± 0.7 | 0.987 | **30.2 ± 0.9$^{AB,\wedge}$** | **4.8 ± 0.6$^{CDE}$** | **2641 ± 753.7** | **0.996** |
| | $Na_2SO_4$ | 56.9 | 21.3 ± 1.0 | 5.9 ± 1.2 | 0.984 | **26.7 ± 1.0$^{BC}$** | **9.7 ± 0.9$^{BC,\wedge}$** | **537.1 ± 97.3** | **0.998** |
| | $NaHCO_3$ | 57.7 | 32.2 ± 2.0$^{A,\wedge}$ | 20.6 ± 4.3$^{A,\wedge}$ | 0.987 | NA | | | |
| | KCl | 12.7 | 29.1 ± 1.0$^{AB}$ | 7.6 ± 0.8$^{BCD,\wedge}$ | 0.998 | NA | | | |
| | $K_2SO_4$ | 5.7 | 27.3 ± 0.8$^{B}$ | 2.3 ± 0.2$^{DE,\wedge}$ | 0.997 | NA | | | |
| | $KHCO_3$ | 9.4 | 31.1 ± 0.8$^{AB}$ | 12.0 ± 0.7$^{B,\wedge}$ | 0.999 | NA | | | |
| | $CaCl_2$ | 60.0 | 24.5 ± 1.3 | 0.5 ± 0.2 | 0.941 | **29.5 ± 0.4$^{AB,\wedge}$** | **1.1 ± 0.1$^{DE,\wedge}$** | **377.8 ± 29.1** | **0.999** |
| | $CaSO_4$ | ≥14.5[c] | 29.9 ± 0.8$^{AB,\wedge}$ | 0.8 ± 0.1$^{DE}$ | 0.997 | NA | | | |
| | $MgCl_2$ | 37.0 | 28.1 ± 0.7 | 0.5 ± 0.1 | 0.988 | **29.9 ± 0.7$^{AB,\wedge}$** | **0.6 ± 0.1$^{E,\wedge}$** | **855 ± 275.4** | **0.996** |
| | $MgSO_4$ | 38.5 | 22.1 ± 0.5$^{C,\wedge}$ | 2.2 ± 0.3$^{DE,\wedge}$ | 0.993 | NA | | | |

[a] 96 h-LC50 for FHM from Mount et al. (1997).
[b] 24 h-LC50 for RBT from Durand-Hoffman (1995).
[c] The LC50 was above the solubility limit.

Oakton WD-35801 electrode (Table S3). During the exposures, the solutions were maintained at the acclimation temperatures (RBT:11 ± 1 °C, GF: 18 ± 1 °C).

An additional test on whether the TEP had any electrogenic component (i.e. caused by the presence of active pumps which transport net charge across epithelia) was performed using Cortland physiological saline for freshwater fish (Wolf, 1963) without added glucose. Exposure to this solution was expected to eliminate all ionic diffusion gradients between the extracellular fluid of the fish and the environment, so that if a TEP persisted, it would be of electrogenic origin (Potts, 1984).

### 2.3. Transepithelial potential (TEP) measurements

The preparation of fish and the measurement of TEP followed the procedures in Wood et al. (2020). Briefly, RBT and GF were fasted for 48 h before surgery, and then anaesthetized with neutralized MS-222 (0.2 mg/L, Sigma-Aldrich). The gills of RBT were irrigated with a continuous flow of the MS-222 solution to avoid hypoxic stress, but this was not required for GF as they can tolerate brief hypoxia. A saline-filled polyethylene PE50 catheter (3–4 cm, Clay Adams™, Becton Dickinson) was inserted into the peritoneal cavity (extracellular fluid), secured in place by an outer PE160 sleeve and silk sutures, and sealed with a pin. Surgery was completed within 5 min and the fish were returned to separate enclosures within the same tank of aerated, temperature-controlled acclimation water for recovery (6–12 h) prior to experimentation.

By convention, TEP across the gills is measured as the inside voltage relative to the outside voltage (ambient water) as 0 mV (Potts, 1984). Voltage measurements were made via Ag/AgCl electrodes (World Precision Instruments) connected to a pHM 82 pH meter (Radiometer-Copenhagen, which served as a high impedance voltmeter. The electrodes were connected to the measurement points via PE160 tubing filled with 3 M KCl and tipped with PE90 filled with 3 M KCl-agar as bridges. The bridge of the reference electrode was inserted into the external solution, and the bridge of the measurement electrode was connected to the saline-filled intraperitoneal catheter. Absolute TEP of an individual fish at a given moment was the voltage difference measured between the intraperitoneal catheter and the exposure solution. A single TEP result was an average of three separate measurements, each corrected by an intervening junction potential measurement.

The baseline TEP was first measured in triplicate while the fish was in acclimation water alone. All subsequent TEP measurements were compared to this baseline to calculate the change of TEP (ΔTEP). Fish were then transferred gently to the first experimental solution (the lowest concentration) and allowed to stabilize for 3 min before the measurement of TEP, again in triplicate. The measurement series continued by transferring the fish to solutions of increasing salt concentrations until the highest concentration. A total of 6–12 RBT and 6–8 GF were used as replicates for each salt exposure series. New salt solutions were used for each fish.

### 2.4. Plasma ion analysis

After the completion of TEP measurements for each salt, fish were euthanized in a lethal concentration of neutralized MS-222 (0.8 g/L), briefly rinsed in clean water, and blotted dry. Blood was collected by caudal puncture with a modified 100-μL gas-tight syringe (Hamilton) that had been pre-rinsed with saline containing 1000 i.u./mL of lithium heparin (Sigma-Aldrich). The collected blood was cooled in ice before being centrifugation (5000G, 2 min). Plasma was decanted and stored at −20 °C.

The concentrations of Na and K in plasma were measured by atomic absorption spectroscopy (SpectroAA220 FS, Varian) in flame mode equipped with a Varian hollow cathode lamp for Na/K, and calibrated with commercial standards (Fisher-Scientific). A 5000x dilution with de-ionized water brought plasma samples within the optimal range of the standard solutions.

### 2.5. Statistical analysis

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 (all data passed) and one-way Analysis of Variance tests were performed for comparisons of the baseline TEP, ΔTEP values at LC50 and plasma concentrations. Tukey's post-hoc HSD test was used to identify specific differences among several groups, or Student's t-test for comparisons between species ($p < 0.05$).

The experimental means of ΔTEP as a function of salt exposure concentration were fitted by non-linear regression (with the R function *nls()* for nonlinear least squares) to two possible models, illustrated in Fig. S1, as empirical constructs. Equation (2) is the Michaelis-Menten (M-M) model often employed in enzyme and transport kinetics, which was used to successfully fit most of the salt series ΔTEP data in Wood et al. (2020):

$$\Delta TEP = \frac{TEP_{max} \times [Salt]_o}{K_m + [Salt]_o} \quad (2)$$

Equation (3) is a modified M-M equation that takes substrate inhibition into account, fitted with the analytical method using R described by Huitema and Horsman (2018):

$$\Delta TEP = \frac{TEP_{max} \times [Salt]_o}{K_m + [Salt]_o \times \left(1 + \frac{[Salt]_o}{K_s}\right)} \quad (3)$$

where: $\Delta TEP$ = change in TEP (mV) relative to baseline TEP in background water.

$\Delta TEP_{max}$ = maximum change in TEP (mV) relative to baseline TEP.

$[Salt]_o$ = external salt concentration (mmol/L).

$K_m$ = affinity constant (mmol/L) representing the $[Salt]_o$ associated with 50% of $TEP_{max}$.

and in Equation (3):

$K_s$ = constant (mmol/L) accounting for the inhibition of the substrate.

For most of the salts, the data fitted well to only one of the equations. However, when a salt data set fitted both models, the model with the higher $R^2$ value was used.

## 3. Results

Baseline TEP values ranged from −37.5 to −4.0 mV in RBT, and −39.5 to −3.3 mV in GF, with averages of −22.1 ± 0.7 mV (N = 81) and −18.7 ± 1.0 mV (N = 68) in RBT and GF, respectively (Fig. S2a). These baseline TEP were significantly different between the two species ($p < 0.01$). Under acute transfer from freshwater to an isotonic physiological saline, both RBT and GF increased their TEP to values not significantly different from zero (Figs. S2b and S2c), demonstrating that there was no electrogenic component in either species.

Both RBT (Fig. 1) and GF (Fig. 2) exhibited a progressively increasing ΔTEP during exposure to increasing concentrations of salts, until the response reached saturation (i.e. a hyperbolic function), or sometimes slightly declined. In most of cases, the relationship started to plateau right before or at the range between the 96 h- and 24 h-LC50s.

For most salts, the M-M model (Equation (2)) provided an excellent fit ($R^2$ = 0.941–0.999) but for a few salts where ΔTEP dropped at higher concentrations ($CaCl_2$ in both species, and NaCl, $Na_2SO_4$, and $MgCl_2$ in goldfish), the modified M-M model with substrate inhibition (Equation (3), Fig. S1)) provided a better fit ($R^2$ = 0.996–0.999) as shown in Figs. 1 and 2. The ΔTEP response versus salt concentration curves with the M-M model were generally significant ($p < 0.01$), with individually significant Michaelis constants ($K_m$) and $\Delta TEP_{max}$ values ($p < 0.05$) except for the $CaCl_2$ response for both species.

To better separate the points at lower concentrations, the data from Figs. 1 and 2 have been replotted on a logarithmic concentration scale in Figs. S3 and S4. These relationships remain curved, illustrating that they were originally hyperbolic, not log-linear functions.

The theoretical maximum ΔTEP ($\Delta TEP_{max}$) resulting from the best-fit models for each salt ranged from 18.5 to 34.3 mV for RBT, and from 22.1 to 32.2 mV for GF (Table 1). There were significant differences in both $\Delta TEP_{max}$ and $K_m$ between both salts and species of fish (2-way ANOVA, $p < 0.001$). In RBT, significantly higher $\Delta TEP_{max}$ was found in the $KHCO_3$ response (Tukey test, $p < 0.05$) (Table 1). Apart from $Na_2SO_4$, KCl, $K_2SO_4$ and $KHCO_3$, RBT exhibited significantly lower $\Delta TEP_{max}$ than GF (Table 1). The Michaelis constants ($K_m$) ranged from 0.5 to 6.3 mM for RBT and from 0.6 to 20.6 mM for GF. GF exhibited significantly higher $K_m$ in response to most salts except for NaCl and $CaSO_4$ (no significant difference) and for $MgCl_2$ (significantly lower $K_m$) (Table 1). GF had extraordinarily high $K_m$ in response to the $HCO_3^-$ salts, at least 90% higher than the highest $K_m$ of RBT (in $NaHCO_3$). These $K_m$ values were the only ones which were higher than the 96 h-LC50 of the respective salt (Table 1 and Table S1).

The $K_m$ ranges for Na and K salts (1.1–20.6 mM) were generally higher than for Ca and Mg salts (0.5–2.2 mM) (Table 1, Fig. S5). Although the logarithm of $K_m$ had an overall positive relationship with the logarithm of 24-h LC50, only the regressions for Na and K salts were significant ($R^2$ = 0.56 for RBT and 0.10 for GF) (Fig. S5).

A more detailed comparison of ΔTEP responses at lower concentrations of the major salts was carried out by plotting each response as a percentage of the ΔTEP at the 24-h LC50 for that salt (Fig. 3). The effect of common cations on the rise in ΔTEP (Fig. 3(R1–R4, G1–G4)) was clearly more important than the effects of common anions (Fig. 3(R5–R7, G5–G7)), as seen by the larger difference among the latter curves. From the plots grouped by anions, it was clear that the toxicity of individual salts (determined by the LC50 values) was reflected in the steepness of the curve. For instance, KCl and $K_2SO_4$ (Fig. 3(R5–R6, G5–G6)) which are the most toxic among the salts exhibited the steepest initial slopes.

A key finding is the consistency in the ΔTEP values at the LC50s. In RBT, the ΔTEP values at the 24-h LC50 concentrations were not significantly different among the ten salts (Fig. 4). In GF, values were also fairly constant, but a lower ΔTEP was seen for $KHCO_3$ compared to NaCl, $CaSO_4$ or $MgCl_2$ at their respective LC50 levels (Fig. 4). Nevertheless, the largest fold variations in ΔTEP at LC50 among all the salts were only 1.6-fold for RBT (15.9 mV in $MgSO_4$ vs 26.1 mV in $Na_2SO_4$) and 2.1-fold for GF (13.8 mV in $KHCO_3$ vs 29.0 mV in $CaSO_4$). Therefore, there was a great consistency in ΔTEP at the LC50 for different salts within a species in the face of a 19-fold (RBT) to 25-fold (GF) variation in the absolute LC50 concentrations among the salts. Comparing between species, RBT were significantly higher in ΔTEP at the 24-h LC50 in $KHCO_3$ exposures, while GF were significantly higher in ΔTEP at the 24-h LC50 in $CaSO_4$ and $MgCl_2$ exposures (Fig. 4).

In both species, the plasma Na concentrations after exposure to the whole series of increasing salt concentrations did not vary from the mean of the relevant procedural control (Fig. 5a). This was also true for the mean plasma K concentration in GF (Fig. 5b). However, in RBT, the mean plasma K concentration after exposure to the series of NaCl and $KHCO_3$ salts significantly increased by 66% and 60%, respectively (Fig. 5b). In GF, the highest two to three plasma Na

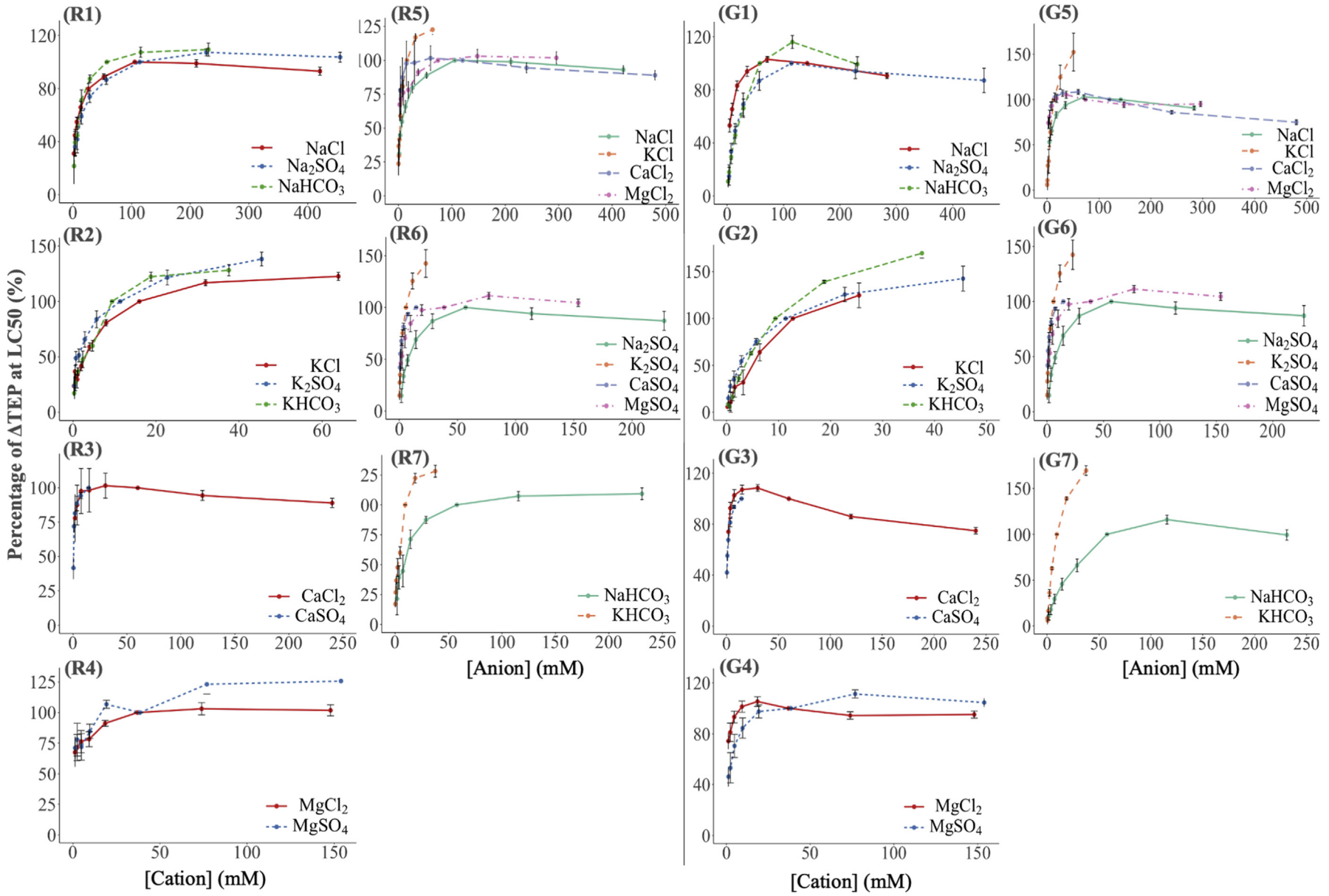


**Fig. 3.** Percentage of the change in transepithelial potential (ΔTEP) at the LC50 concentration in RBT (R1 − R7) and GF (G1 − G7) plotted as a function of the cation concentration (mM) for (1) $Na^+$, (2) $K^+$, (3) $Ca^{2+}$, and (4) $Mg^{2+}$, and plotted as a function of the anion concentration (mM) for (5) $Cl^-$, (6) $SO_4^{2-}$, and (7) $HCO_3^-$. Means ± SEM, N = 6–12.

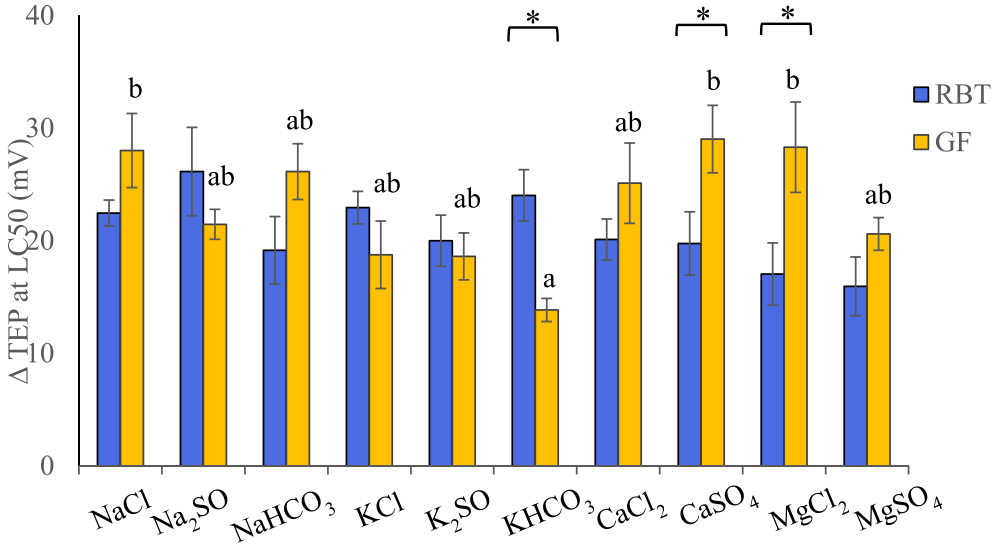


**Fig. 4.** Changes in transepithelial potential (ΔTEP) at the 24-h LC50 (referenced from fathead minnow) in rainbow trout (RBT) and goldfish (GF) for 10 different salts. Means ± 1 SEM (N = 6–9). Among different salts, the ΔTEP values at the LC50 were not significantly different in RBT ($F_{(9,66)} = 1.784$, $p = 0.088$) but differences existed in GF, which are shown by different letters on each bar (One-way ANOVA, $F_{(9,55)} = 3.709$, $p = 001$; and Tukey test $p < 0.05$). Asterisk (*) indicates significant differences in ΔTEP at the LC50 between RBT and GF for the same salt (*t*-test, $p < 0.05$).

and K concentrations were associated with the respective cations (Na or K) of the salts that were tested.

## 4. Discussion

### 4.1. Overview

The TEP responses during acute major ion exposures of RBT and GF provided qualified empirical support for the MIT model (EPRI, 2018), in accord with our first objective. The lack of electrogenic components to the TEP in both species allowed direct interpretation of the TEPs as diffusion potentials, as well as comparison to the ~~r~~esults of Wood et al. (2020). Both RBT and GF responded similarly to the FHM, CC, and BG in Wood et al. (2020) in terms of the M-M relationships and the flattening of ΔTEP at major ion concentrations close to the 96-h LC50 of FHM. As discussed subsequently, this flattening response is a point of discrepancy from the predictions of the MIT model. Nevertheless, the key conclusion is that within a species, the ΔTEP at the LC50 is remarkably uniform among different salts, despite the great variation in the absolute salt concentrations causing the response, and is therefore a suitable endpoint for toxicity prediction in the MIT model (EPRI, 2018). Despite the much lower water [$Ca^{2+}$] in the present study, the same basic principles were seen, answering our second objective, but we caution that confounding factors such as the differences in fish species and in holding conditions with the Wood et al. (2020) study limit conclusions that can be made as to the effects of water hardness. With respect to the third objective, $NaHCO_3$ and $KHCO_3$ presented some exceptions to the general patterns of the other eight salts, effects perhaps associated with their actions on water pH. For our final objective, the relative constancy of plasma $Na^+$ and $K^+$ concentrations at the end of the exposures provided support for the assumption of unchanged values in the MIT model.

### 4.2. ΔTEP response patterns

The qualitatively similar way in which ΔTEPs reached their maxima as the salt concentrations approached the LC50s showed that the depolarization responses caused by major ions were similar among different species, even in the much softer, ion-poor water of the present study. This occurred despite obviously higher deflections in TEP, where the salt-specific ΔTEPs at LC50 ranged from +13.8 to +29.0 mV (Fig. 4), compared to the three species tested in moderately hard water where the highest mean ΔTEP at the LC50 was only +8.9 mV (Wood et al., 2020). The larger deflections may result from the more negative baseline TEP (means of −18.7 mV in GF, −22.1 mV in RBT; Fig. S2a) in the softer water compared to higher baseline TEP (−6.8 mV in FHM, −5.3 mV in CC, and −2.9 mV in BG) in moderately hard water (Wood et al., 2020), though we cannot eliminate species differences as a contributing factor. The internal fluids of different species have only minimal variation in ionic composition, thus, when their external water is more dilute, the higher electrochemical gradients across the membrane will lead to a more negative baseline TEP by Equation (1). This assumes that the TEP is mainly driven by the differential diffusion of cations versus anions as is generally believed (Eddy, 1975; Potts, 1984; Wood and Grosell, 2008), and is captured in the MIT model (EPRI, 2018). In agreement with Wood et al. (2020), the TEP responses of both RBT and GF were largely driven by the cation concentrations rather than by the anion concentrations (Fig. 3), in line with the cation-dependent toxicities found in recent studies (Erickson et al., 2017; Mount et al., 2016).

There were several instances (asterisks in Figs. 1 and 2) where ΔTEP reached a maximum and then declined with further increases in salt concentration. In these cases, the data were better fitted by the modified (Equation (3)) rather than by the standard M-M model (Equation (2)). If we use ΔTEP as an indicator of toxicity, this could indicate that for GF 4 × the LC50 is less toxic than 1 × the LC50 for $CaCl_2$, which seems impossible. This anomaly occurs well above levels of environmental relevance where the fish is going to die anyway, and would not have been predicted by the MIT model. This phenomenon was more prevalent in the GF than in the RBT, and was often associated with $Cl^-$ salts. Indeed, Eddy (1975) reported this exact same phenomenon with $Cl^-$ salts in GF. The likely explanation is that at very high salt concentrations where the cation transport has already reached saturation, the chemical gradient for the anion ($Cl^-$) becomes high enough to cause sufficient $Cl^-$ influx to attenuate the ΔTEP. Interestingly the

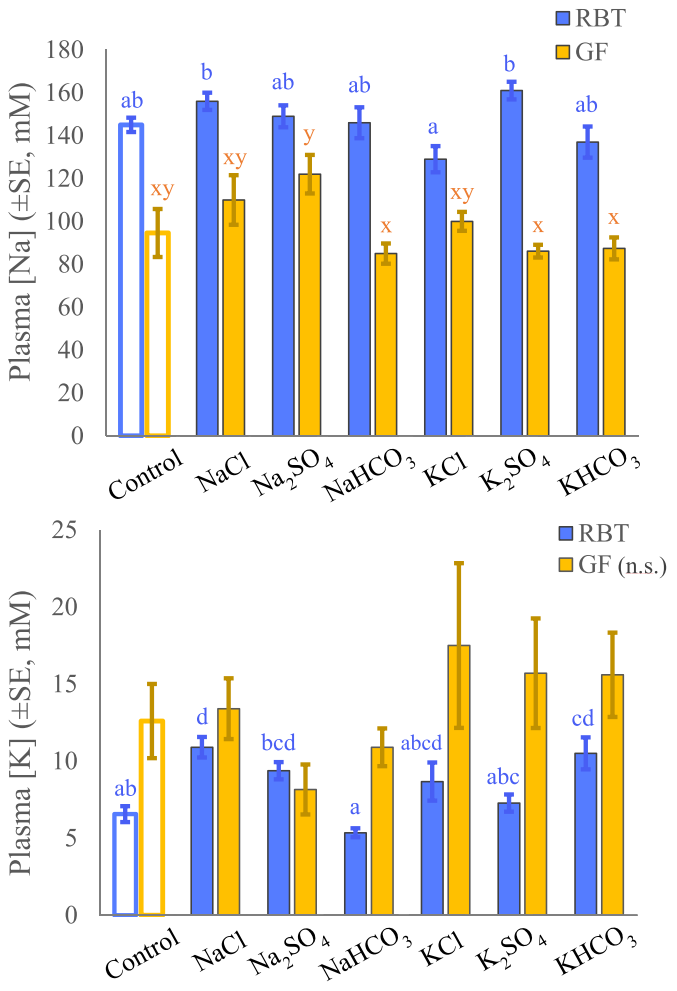


**Fig. 5.** Plasma (a) sodium and (b) potassium concentrations (mM) in rainbow trout (RBT) and goldfish (GF) exposed to major sodium and potassium salts. The solid bars represent the values of fish sampled after they had been serially exposed to increasing concentrations of the salts of sodium and potassium, and used for TEP measurements, as in Figs. 1 and 2. The open bars on the far left represent the values of procedural control fish which had undergone the same cannulation and handling but without exposure to major salts. Bars not sharing the same letter are significantly different within a species (One-way ANOVA and Tukey's test, $p < 0.05$); n.s.: not significantly different. Means ± SEM, N = 6.

phenomenon was not seen in FHM, CC, and BG (Wood et al., 2020). Again, we cannot eliminate species differences, but the anticipated lower overall permeability due to the higher [$Ca^{2+}$] in water (Table S2), as discussed below, may have prevented the phenomenon. Note that the much lower baseline TEP values in RBT and GF did not change the consistency (within a species) of ΔTEP at the LC50s among the major salts, in contrast to the diverse LC50 concentrations (Fig. 4). This provides support for the assumption of the MIT model (EPRI, 2018) that depolarization at the gill is representative of toxicity. The reasons why these consistent depolarizations are associated with ultimate lethality remain unknown, but may be related to internal ionic or acid-base disturbances as postulated by Wood et al. (2020).

It should be noted that the MIT model (EPRI 2018) predicts that ΔTEP should continue to increase as salt concentration increases, in contrast to the flattening response observed in our data. The reason for this discrepancy is unknown. The most likely explanation is that the actual ΔTEP deviates from the theoretical ΔTEP because this occurs close to the LC50 - i.e. at the point where toxicity is occurring. The theory behind the GHK and Spangler equations, from which the MIT model is derived, is based on normally functioning, healthy cell membranes, and this may no longer be the case when toxicity occurs. The other possibility is that at high concentrations, channels may saturate, ion-pairing may occur, and membrane surface charge effects may be induced, all of which could contribute to the asymptotes that we observed; none of these were taken into account in the original GHK and Spangler formulations (Alvarez and Latorre, 2017; French and Adelman, 1976; McLaughlin, 1977; Syganow and Von Kitzing, 1999). Nevertheless, the region of ΔTEP saturation is generally close to the LC50, so this may not matter in terms of the utility of the EPRI model as tool to predict toxicity.

Wood et al. (2020) reported strong positive relationships (on a log-log scale) between the Michaelis constants *Km* and the corresponding LC50s in FHM, CC, and BG in moderately hard water, and interpreted this as evidence that their ΔTEP measurements were representative of the toxicities of the major salts. However, in the present study, comparable relationships were relatively weak (Fig. S5). The reasons for this discrepancy may include species differences, the much softer water, and the reliance on LC50 values that were not species-specific.

### 4.3. The influence of water [$Ca^{2+}$]

Low water [$Ca^{2+}$] is recognized as a key factor in making the absolute TEP in freshwater fish more negative, while high water [$Ca^{2+}$] raises it (Eddy, 1975; McWilliams and Potts, 1978; Wood et al., 1998; Wood and Grosell, 2008). These effects occurred steeply over a narrow concentration range (Fig. 3(R3, G3), a phenomenon also reflected in the very low *Km* values (i.e. high affinities) for the two $Ca^{2+}$ salts in these soft-water acclimated fish (Table 1). In contrast, FHM, CC, and BG acclimated to moderately hard water (7-fold higher [$Ca^{2+}$], Table S2), exhibited 1.5- to 50-fold higher *Km* values for the same salts. Elevated water [$Ca^{2+}$] also protects against the toxicity of a wide variety of substances, including major cations (e.g. $Na^+$, $Mg^{2+}$ and $K^+$ in *Ceriodaphnia dubia* and FHM (Erickson et al., 2017; Mount et al., 1997)) and certain salts (e.g. $Na_2SO_4$ and NaCl in RBT (Elphick et al., 2011a, b)). External $Ca^{2+}$ ions generally reduce the permeability of fish gills to many substances including strong ions (Cuthbert and Maetz, 1972; Hunn, 1985; McDonald and Rogano, 1986). $Ca^{2+}$ appears to act by tightening the paracellular junctions between the gill cells, and by titrating the negative charge on these pathways (Evans et al., 2005; Potts, 1984). While gills become less permeable overall, their relative anion-to-cation permeability increases, both of which make the TEP increase. Thus, at the same time as $Ca^{2+}$ protects against the toxicity of other ions, in itself it is potent in causing depolarization. Likely the varying balance of these dual, opposing actions explains the observations that increased water hardness may either ameliorate the toxicity of other major ions, or contribute to overall toxicity (Bogart et al., 2019; Davies and Hall, 2007; Elphick et al., 2011a, b; Erickson et al., 2017; Mount et al., 2016).

### 4.4. Rainbow trout versus goldfish

Despite generally similar responses between the two species, there were some notable differences. Firstly, it was apparent that divalent cations were handled differently, as evidenced by the significantly lower $\Delta TEP_{max}$ in RBT than in GF for all of the Ca and Mg salts (Table 1). Secondly, for RBT, the higher $\Delta TEP_{max}$ in the KCl exposures (28.2 ± 2.0 mV) compared with the NaCl exposures (21.9 ± 0.6 mV) could be related to the drop in $Cl^-$ influx under KCl exposure, but not under NaCl and $MgCl_2$ exposures, seen in this species (Kersetter and Kirschner, 1972). Assuming that entrance of

$K^+$ is mainly gradient-driven by diffusion, as external KCl increases, a low $Cl^-$ influx (or even net $Cl^-$ efflux) would make the TEP become more positive. This may also be related to the lesser occurrence of a decline in ΔTEP at high concentrations of $Cl^-$ salts in RBT, suggesting lower permeability to $Cl^-$. However, we are aware of no comparable flux measurements with these salts in GF where the $\Delta TEP_{max}$ values to NaCl and KCl were similar to one another (Table 1). Surprisingly, although RBT are much more tolerant to seawater (USEPA, 1988; Schofield et al., 2006), their *Km* value for NaCl did not differ significantly from GF. Furthermore, *Km* values were lower (i.e. affinities were higher) in RBT than in GF for eight salts (significant in five, Table 1). However, for $MgCl_2$ the *Km* was significantly higher (i.e. lower affinity) than for GF. *A priori*, only the latter difference would seem appropriate for greater seawater tolerance. The opposite occurred for $MgSO_4$, with a significantly lower *Km* in RBT than in GF, suggesting some differences between the two species in the regulation of the anions. $SO_4^{2-}$ appears to be well-regulated at fish gills, but has not been well studied (Griffith et al., 2020). Interestingly, $MgSO_4$ has been reported to increase $Na^+$ outflux but not influx in the GF (Cuthbert and Maetz, 1972), potentially causing a more negative TEP and thus the significantly lower $TEP_{max}$ in this species (Table 1).

### 4.5. ΔTEP responses to bicarbonate salts

We are aware of no comparable TEP data in freshwater fish for bicarbonate salts, apart from a single experiment of Eddy (1975) showing TEP increasing with elevations in $NaHCO_3$ concentration, in a background of de-ionized water. Comparison of the present ΔTEP profiles for $NaHCO_3$ (Fig. 3(R1, G1)) with responses to other $Na^+$ salts, shows that they coincide very closely. Similarly, when the ΔTEP profiles for $KHCO_3$ are compared with responses to other $K^+$ salts (Fig. 3(R2, G2)), they again agree well. These close agreements support the general interpretation that these responses are cationic diffusion potentials, in accord with evidence that $HCO_3^-$ permeability of the teleost gill is very low relative to $Na^+$ permeability (Perry et al., 1982; Wood et al., 2012). However, the unusual feature of the $HCO_3^-$ salt responses is their positions relative to LC50 thresholds. In contrast to most of the other salts, ΔTEP kept increasing well beyond the 96-h LC50; this difference was apparent but less clear with respect to the 24-h LC50 as a reference point (Figs. 1 and 2). This suggests that toxicity may be exacerbated by other factors.

Very probably, these other factors are related to the increase in water pH to about 8.4–8.5 caused by the $HCO_3^-$ salts, an effect that does not occur with the other salts (Table S3). One recent survey of $NaHCO_3$ toxicity to a range of freshwater organisms attributed toxicity to the $HCO_3^-$ anion, rather than to the cation (Harper et al., 2014), and another found $NaHCO_3$ to be more toxic than a seawater salt mixture at comparable salinity or conductivity (Hills et al., 2019). This elevated pH range is certainly within the range of tolerance of most teleosts. Indeed, at an inspired pH of 8.5 in soft water, the acidification provided by both an electrogenic $H^+$ pump on the gill epithelium (Lin and Randall, 1991) and metabolic $CO_2$ production (Playle and Wood, 1989) can lower water pH in the gill micro-environment of the RBT by more than 1.0 pH unit (Playle and Wood, 1989). Nevertheless, this alkaline pH is still sufficient to interfere with acid-base balance, ammonia excretion, and ion transport at the gills (Wright and Wood, 1985; Wilkie and Wood, 1996). Perhaps these effects of high pH exacerbate the stresses reflected in the ΔTEP response. While this pH effect is a theoretical complication, the ΔTEP responses at the LC50 still lie within the ranges of the other salts (Fig. 4), so toxicity values predicted by the MIT model should still have validity.

### 4.6. Plasma $Na^+$ and $K^+$ responses

As a predictive tool, the MIT Model uses the initial ion concentrations (i.e. $[ion]_i$ in Equation (1)) in the blood plasma of the unimpacted animal (EPRI, 2018). If these were to change greatly during exposure, the model predictions would be in error. The present measurements (Fig. 5) of relatively unchanged plasma $[Na^+]_i$ and $[K^+]_i$ after about an hour of exposure to progressively increasing salt concentrations as high as 4 times the LC50 allays these concerns to some extent. $[Na^+]_i$, the internal ion that has the greatest effect on model predictions, remained unchanged for all 6 $Na^+$ and $K^+$ salts, and $[K^+]_i$, the second most influential internal ion, varied by less than 2-fold. However, this exposure time might not be adequate to represent longer exposures where changes in plasma ions have been observed (e.g. Grant et al., 2010; Salati et al., 2011). Future studies should assess these and other plasma ions over a time scale relevant to acute toxicity measurements (e.g. 96 h). A recent study on daphnia showed significant changes in hemolymph ions with salt exposures (Morris et al., 2021).

### 4.7. Future perspectives

Our results with TEP measurements on two fish species adapted to very soft water are in accord with earlier observations on three other species in moderately hard water (Wood et al., 2020). The same principles now apply in four different Orders (Cypriniformes, Siluriformes, Perciformes, Salmoniformes), the latter providing the first examination of a euryhaline fish species (RBT). Overall, the results provide qualified support for the MIT model (EPRI, 2018) in freshwater fish. The reasons for the observed saturation of the ΔTEP responses with concentration *versus* the non-saturation predicted by the MIT model should be explored. As TEP is a diffusion potential and internal plasma ion concentrations are generally uniform amongst freshwater fish, the differences in mean baseline TEP values amongst species (varying from − 3 mV in BG to −22 mV in RBT) and in ΔTEP at the LC50 (varying from 4 mV in BG to about 25 mV in trout and goldfish) must reflect species-dependent and/or water chemistry-dependent differences in the relative permeabilities of the gill epithelia to the different ions. These differences are well-documented in the literature and explain why different fish have very different rates of compensating ion uptake at the gills, and why water hardness and the levels of other major ions have such large effects on TEP. Indeed, relative ion permeabilities are in fact the major variables that are manipulated in the MIT model in order to accommodate species differences and to fit TEP changes to their toxicity data. These manipulations should be made explicit by incorporating additional mechanistic knowledge into the model, not just by fitting. For this, TEP and toxicity data on a much wider range of species, especially aquatic invertebrates, and a variety of waters are needed. Understanding ΔTEP responses in mixtures of multiple ions and how this relates to toxicity is the next major challenge. Future studies should also evaluate how pH changes affect both major ion toxicity and the ΔTEP response profiles, because freshwater alkalinization is often coupled with salinization (Kaushal et al., 2018). Resolution of these uncertainties will improve the ability of the MIT model to generate future environmental guidelines for major ions, and for providing risk assessments for unusual salt mixtures. The relatively quick and easy ΔTEP measurements provide integrative measures of salt toxicity based on a physiological parameter relevant to the mechanism(s) of impact. As such, they may prove useful as an intermediate validation step in checking and refining the model predictions, prior to embarking on costly and time-consuming toxicity testing.

## Declaration of competing interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.116699.

## Author statement

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