

The potential for salt toxicity: Can the trans-epithelial potential (TEP) across the gills serve as a metric for major ion toxicity in fish?

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ABSTRACT

An emerging Multi-Ion Toxicity (MIT) model for assessment of environmental salt pollution is based on the premise that major ion toxicity to aquatic organisms is related to a critical disturbance of the trans-epithelial potential across the gills (Δ TEP), which can be predicted by electrochemical theory. However, the model has never been evaluated physiologically. We directly tested key assumptions by examining the individual effects of eight different salts (NaCl, Na₂SO₄, MgCl₂, MgSO₄, KCl, K₂SO₄, CaCl₂, and CaSO₄) on measured TEP in three different fish species (fathead minnow, *Pimephales promelas* = FHM; channel catfish, *Ictalurus punctatus* = CC; bluegill, *Lepomis macrochirus* = BG). A geometric concentration series based on previously reported 96-h LC50 values for FHM was used. All salts caused concentration-dependent increases in TEP to less negative/more positive values in a pattern well-described by the Michaelis-Menten equation. The Δ TEP responses for different salts were similar to one another within each species when concentrations were expressed as a percentage of the FHM LC50. A plateau was reached at or before 100 % of the LC50 where the Δ TEP values were remarkably consistent, with only 1.4 to 2.2-fold variation. This relative uniformity in the Δ TEP responses contrasts with 28-fold variation in salt concentration (in mmol L⁻¹), 9.6-fold in total dissolved solids, and 7.9-fold in conductivity at the LC50. The Michaelis-Menten *K_m* values (salt concentrations causing 50 % of the Δ TEP_{max}) were positively related to the 96-h LC50 values. Δ TEP responses were not a direct effect of osmolarity in all species and were related to specific cation rather than specific anion concentrations in FHM. These responses were stable for up to 24 h in CC. The results provide strong physiological support for the assumptions of the MIT model, are coherent with electrochemical theory, and point to areas for future research.

1. Introduction

The damaging effect on aquatic ecosystems of elevated major ion concentrations (Na⁺, K⁺, Ca²⁺, Mg²⁺, Cl⁻, SO₄²⁻, HCO₃⁻ / CO₃²⁻) in surface waters is a problem of growing concern (Goodfellow et al., 2000; Findlay and Kelly, 2011; Cañedo-Argüelles et al., 2013, 2016; Herbert et al., 2015). In some contaminated sites, individual ion levels may even exceed those in sea water. Sources of major ions include general urbanization (Estévez et al., 2019), saltwater intrusion due to sea level rise (Venâncio et al., 2019), oil-field saline discharges (Boelter

et al., 1992), irrigation runoff from agriculture (Smedema and Shiati, 2002), mining (Kennedy et al., 2005; Pond et al., 2008), road de-icing salts (Findlay and Kelly, 2011; Moore et al., 2019), and fracking fluid spills (Blewett et al., 2017). As a result, there has been a concerted research effort into the toxicological effects of elevations of major ions and their interactions (Mount et al., 1997; Tietge et al., 1997; Griffith et al., 2012; Cormier and Suter, 2013; Cormier et al., 2013a, b; Mount et al., 2016; Erickson et al., 2017, 2018). While early emphasis was placed on anion toxicity, more recent analyses have attributed toxicity to the cationic components of major salts. Nevertheless, the assessment

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framework for major ion toxicity remains fragmentary and incomplete. For example, osmolarity, conductivity, total dissolved solids (TDS), salinity, and the concentrations of particular cations and anions have all been suggested as measures for environmental regulations to limit major ion concentrations in freshwaters (USEPA, 2011; Cormier et al., 2013a; EPRI, 2018), but there is no general agreement. Only Cl^- and SO_4^{2-} anions are commonly listed in the water quality criteria in different countries (e.g., USEPA, 1988; CCME, 2011; Elphick et al., 2011a, b) and some limited attention has been paid to assessments framed in terms of salinity, TDS, or conductivity in regional criteria (USEPA, 2016). In addition, in some regional jurisdictions in North America, there have been some considerations of background concentrations of Cl^- and SO_4^{2-} , as well as the influence of hardness (e.g. Davies and Hall, 2007; Elphick et al., 2011a, b; Bogart et al., 2019). However, in general, none of these measures take into account the composition of the major ions comprising the pollutants or the known differences in toxicity of the major ions. In part, this is due to a lack of understanding of the physiological mechanism(s) underlying the toxicity of major ions, either alone or in combination, and consequently the lack of a metric tied to the mechanisms of aquatic toxicity, which should provide the most robust basis for assessment.

Clearly, risk assessment approaches are needed that take into account the differential toxicity of various ions (Cañedo-Argüelles et al., 2016; Schuler et al., 2019). Over the last few years, the Electric Power Research Institute (EPRI) has developed a predictive toxicity model that attempts to do this (EPRI, 2018, and previous versions referenced therein). Its most recent iteration, the “MIT” (Multi-Ion Toxicity) model (EPRI, 2018), is based on the knowledge that major ions differentially disturb electrical gradients across biological membranes, and the assumption that this disturbance, primarily at the gills of freshwater animals, ultimately leads to toxicity and death. Therefore, the model is based on the premise that a certain depolarization (i.e., an increase of a few mV) of the trans-epithelial potential (TEP) across the gills is predictive of incipient mortality, and that this depolarization can be predicted by the Goldman-Hodgkin-Katz Equation:

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

where:

R: universal gas constant, which is 8.314 joules/mole/°K

T: absolute temperature, in °K (i.e. Temperature in °C + 273)

F: Faraday constant, which is 9.649×10^4 Coulombs per mol

TEP: trans-epithelial potential, in volts (equivalent to joules per Coulomb)

p_{ion} : permeability for subscripted ion, in meters per second

$[ion]_o$: ion activity in the external water in mmol L^{-1}

$[ion]_i$: ion activity in the extracellular fluid of the organism (blood plasma or hemolymph) in mmol L^{-1}

or by a more complex extension (Spangler, 1972) of this that considers both divalent and monovalent ions. These equations were originally developed to predict cellular membrane potentials (Goldman, 1943; Hodgkin and Katz, 1949; Pickard, 1976), and are adapted in the EPRI MIT model to predict TEP across the body surface, primarily the gills. In practice, the MIT model substitutes ion concentrations for ion activities in the calculations because of uncertainties about activity coefficients in the internal biological fluids. Sensitivity analyses show that the resulting errors are relatively minor (EPRI, 2018).

The key elements of these equations are the concentrations of the ions in the external water and internal fluids (blood plasma or hemolymph) of the animal, and the relative permeabilities (p) of the gills to each of these ions. Permeability coefficients are not absolute values, but rather relative values. For example, the permeability to Na^+ , Ca^{2+} , or Mg^{2+} may be expressed as a ratio of the permeability to K^+ . EPRI's modeling has used literature sources and unpublished data for measurements of control extracellular fluid composition in various

organisms (EPRI, 2018). However, the relative permeabilities used in the MIT model have not been measured, but rather have been based on the iterative fitting of values to best describe toxicity data from the literature. This fitting allows these ratios to change in different exposure solutions. As a result, the process represents auto-validation rather than independent validation. Another concern is that there is no evidence that a certain disturbance of the TEP is in fact associated with incipient mortality. Thus, the MIT model remains an entirely theoretical exercise based on empirical toxicity data - there is no physiological evidence to support it. Nevertheless, various versions of the model (EPRI, 2018, and previous versions referenced therein) have been very successful in predicting the acute toxicities of major ions, alone and in various mixtures, to fish and daphnids available in extensive data sets in the literature (Mount et al., 1997, 2016; Tietge et al., 1997; Erickson et al., 2017, 2018).

Against this background, the goal of the present study was to use direct measurements of TEP as earlier performed in fish by Potts and Eddy (1973), Eddy (1975), and Wood and Grosell (2008) to test the basic premise of the MIT model in three species of freshwater fish exposed to concentration series of 8 different single salts. The fathead minnow (FHM, *Pimephales promelas*) was chosen because of its status as a model in regulatory toxicology (Ankley and Villeneuve, 2006) and the availability of an extensive data base for acute salt toxicity to this species (Mount et al., 1997). The channel catfish (CC, *Ictalurus punctatus*) was evaluated as an alternative to the fathead minnow because it has similar ionoregulatory physiology (Bentley, 1990) but is more robust for experimentation, and the bluegill (BG, *Lepomis macrochirus*) was tested because it has a fundamentally different ionoregulatory physiology (absence of active branchial Cl^- uptake - Tomasso and Grosell, 2005).

Seven objectives relevant to the MIT model were explored. In accord with the assumptions of the MIT model, we predicted (i) that all salts would disturb the TEP across the gills but that the relationship between the concentration of the salt versus the TEP response would vary greatly depending on the toxicity of the different salts; (ii) that these concentration-response relationships would become much more consistent when concentration was expressed as a percentage of the acute (96-h) LC50, in accord with a common mechanism of toxicity; (iii) that within a species, a certain acute disturbance of the TEP (i.e., ΔTEP relative to baseline) would be predictive of 96-h mortality regardless of the salt; (iv) that this ΔTEP at the LC50 concentration would be much less variable than the molar concentrations, TDS values, or conductivity values of the various salts causing these ΔTEP values. We further analyzed the data to determine (v) whether ΔTEP responses were more strongly associated with the anionic or cationic components of the salts, and whether this related to toxicity. We also tested (vi) whether the osmolarity component of the exposures was responsible for some or all of the effects on TEP, and (vii) whether ΔTEP responses to the different salts were stable over time (24 h).

2. Materials and methods

2.1. Experimental animals and holding

Adult FHM (1.9–5.0 g, $n = 35$) were obtained from a culture at the U.S. EPA Environmental Effects Research Laboratory (Duluth, MN, U.S.A.). Juvenile CC (2.5–12.5 g, $n = 77$) and juvenile BG (4.2–14.8 g, $n = 22$) were obtained from commercial aquaculture (Florida Fish Farms, Center Hill, FL, U.S.A.). All three species were of mixed sex. The fish were acclimated for a minimum of 2 weeks to flowing dechlorinated Miami tap water at the experimental temperature (22–24 °C). The same water was used for background control measurements in all experiments. Miami tap water is a moderately hard water with the following composition in mmol L^{-1} : Na^+ 1.10, Ca^{2+} 0.51, Mg^{2+} 0.13, K^+ 0.08, Cl^- 1.03, SO_4^{2-} 0.36, HCO_3^- 0.68, and DOC (dissolved organic carbon) 0.18, and pH 7.8. All species were fed daily with a mix of

Table 1

A summary of 96-h LC50 values from Mount et al. (1997), together with Michaelis-Menten constants, and R^2 values for the concentration-kinetics curves fitted in Fig. 1. (Means \pm 1 SEM, $N = 6-7$). All relationships were significant ($P < 0.05 - 0.001$) except for KCl in BG where none of the ΔTEP responses were significantly different from zero (n.s.). For constants, A,B,C denote significant differences within a salt among species, whereas W,X,Y,Z denote significant differences within a species among salts. Means sharing the same letter are not significantly different.

Salt 96-h LC50 (mmol L ⁻¹)	Constants	Fathead Minnow (FHM)	Channel Catfish (CC)	Bluegill (BG)
NaCl 109.3	ΔTEP_{max} (mV) Km (mmol L ⁻¹) R^2	$8.82 \pm 0.36^{A,Y}$ $8.87 \pm 1.62^{A,X}$ 0.951	$5.04 \pm 0.68^{B,Y}$ $4.48 \pm 0.41^{B,X}$ 0.982	$3.98 \pm 0.69^{B,X,Y}$ $8.83 \pm 0.94^{A,X}$ 0.983
Na ₂ SO ₄ 56.0	ΔTEP_{max} (mV) Km (mmol L ⁻¹) R^2	$10.19 \pm 0.12^{A,X}$ $7.76 \pm 0.36^{A,X}$ 0.998	$7.60 \pm 0.31^{B,X}$ $8.30 \pm 1.39^{A,W}$ 0.968	$4.04 \pm 0.11^{C,X,Y}$ $5.45 \pm 0.64^{A,X}$ 0.982
MgCl ₂ 22.3	ΔTEP_{max} (mV) Km (mmol L ⁻¹) R^2	$6.19 \pm 0.10^{A,Z}$ $0.81 \pm 0.08^{A,Y}$ 0.980	$5.85 \pm 0.28^{A,Y}$ $0.62 \pm 0.20^{A,Y}$ 0.811	$3.02 \pm 0.27^{B,Y}$ $11.36 \pm 3.19^{B,X}$ 0.951
MgSO ₄ 23.4	ΔTEP_{max} (mV) Km (mmol L ⁻¹) R^2	$7.60 \pm 0.24^{A,Y}$ $1.57 \pm 0.25^{A,Y}$ 0.959	$6.77 \pm 0.26^{A,X,Y}$ $1.73 \pm 0.32^{A,Y}$ 0.946	$4.67 \pm 0.14^{B,X}$ $1.11 \pm 0.18^{A,Y}$ 0.955
KCl 11.8	ΔTEP_{max} (mV) Km (mmol L ⁻¹) R^2	$9.73 \pm 0.82^{A,X,Y}$ $0.82 \pm 0.04^{A,Y}$ 0.997	$7.21 \pm 0.27^{B,X}$ $0.95 \pm 0.17^{A,Y}$ 0.956	n.s. n.s. n.s.
K ₂ SO ₄ 3.9	ΔTEP_{max} (mV) Km (mmol L ⁻¹) R^2	$8.68 \pm 0.42^{A,Y,Z}$ $0.41 \pm 0.09^{A,Z}$ 0.924	$6.13 \pm 0.26^{B,Y}$ $0.23 \pm 0.05^{A,Z}$ 0.902	$3.78 \pm 0.24^{C,X,Y}$ $0.24 \pm 0.05^{A,Z}$ 0.585
CaCl ₂ 41.7	ΔTEP_{max} (mV) Km (mmol L ⁻¹) R^2	$8.68 \pm 0.28^{A,Y,Z}$ $2.23 \pm 0.38^{A,W}$ 0.950	$7.50 \pm 0.27^{B,X}$ $4.37 \pm 0.70^{A,X}$ 0.969	$3.48 \pm 0.59^{C,X,Y}$ $27.58 \pm 13.41^{A,X,Y,Z}$ 0.863
CaSO ₄ > 14.47	ΔTEP_{max} (mV) Km (mmol L ⁻¹) R^2	$7.21 \pm 0.13^{A,Y,Z}$ $0.57 \pm 0.05^{A,Z}$ 0.990	$7.85 \pm 0.36^{A,X}$ 0.96 ± 0.17^A 0.972	$4.12 \pm 0.28^{B,X}$ $0.87 \pm 0.23^{A,Y,Z}$ 0.938

commercial pellets (Aquamax™ sinking pellets, Nestlé Purina Pet Care, St. Louis MO, USA) and flakes (Tetramin™ tropical flakes, Melle, Germany). The fish were fasted for 24 h prior to surgery. All procedures followed an approved University of Miami Animal Care and Use Committee protocol (IACUC #17-150).

2.2. Experimental solutions

All salt stock solutions were made with analytical grade reagents in Miami tap water and experimental solutions were made by dilution of stock solutions into Miami tap water. Therefore, all salt additions were made against a background of Miami tap water. The salts tested were NaCl, Na₂SO₄, MgCl₂, MgSO₄, KCl, K₂SO₄, CaCl₂, and CaSO₄. Conductivities of all solutions were measured with a WTW 3310 conductivity meter (Xylem Analytics, Weilheim, Germany) and total dissolved solid (TDS) values were calculated from the known salt composition.

For uniformity, experimental concentration series for each salt (regardless of the species) were all based on the complete LC50 data set for all 8 salts reported by Mount et al. (1997) for 1 to 7-d old FHM, which are listed in Table 1. There are few salt-specific toxicity data available for the other two species (four values for 96-h LC50 in BG and two for 96-h LC50 in CC, obtained as geometric means from USEPA (1988) and the USEPA ECOTOX, 2020 database (<https://cfpub.epa.gov/ecotox/>), and some of these are from unpublished reports rather than peer-reviewed literature. As these values are reasonably close to those reported by Mount et al. (1997) for FHM, we elected to use the latter values throughout.

For each salt, a geometric series of 8 different concentrations was made. The 6th treatment for any salt was the 96-h LC50 value for FHM from Mount et al. (1997) representing 100 % (Table 1), with 5 others below it, and 2 others above it, in a geometric sequence (3.12 %, 6.25

%, 12.5 %, 25 %, 50 %, 100 %, 200 %, and 400 % of the LC50 value) presented to each fish in increasing order as described subsequently. Note that for CaSO₄, which has low solubility, the 96-h LC50 was reported by Mount et al. (1997) as > 14.5 mmol L⁻¹ (i.e. greater than the solubility limit). We therefore used 13 mmol L⁻¹ as the 6th (and highest) concentration for CaSO₄. An additional series employed a single concentration (900 mmol L⁻¹) of mannitol (analytical grade) as a test of the effect of high osmolarity alone, in the absence of added cations and anions.

2.3. Trans-Epithelial Potential (TEP) measurements

The TEP across the gills of fish is the voltage difference (in mV) between the extracellular body fluids (i.e. blood plasma and interstitial fluid) and the external water. Traditionally, it is expressed as the inside voltage relative to the outside (water) as 0 mV. Earlier studies (Potts and Eddy, 1973; Eddy, 1975; Wood and Grosell, 2008) have demonstrated that the TEP across the gills can be measured via an intraperitoneal catheter, yielding data identical to that obtained by a more invasive vascular catheter. Therefore, intraperitoneal catheters were employed in all experiments.

For surgery, the fish were anaesthetized in neutralized MS-222 (0.3 g L⁻¹, Sigma-Aldrich, St. Louis, MO, U.S.A.). A saline-filled polyethylene PE50 catheter (6–9 cm, Clay Adams™, Becton Dickinson, Franklin Lakes, NJ, U.S.A) was inserted 1–2 cm into the peritoneal cavity and secured in place with an outer PE160 sleeve and silk sutures exactly as described by Wood and Grosell (2008). The catheter was filled with Cortland saline (Wolf, 1963), which mimics the composition of freshwater fish plasma, and sealed with a stain-less steel pin. Fish were then allowed to recover overnight in individual darkened chambers (200-ml polyethylene food containers) served with flowing Miami tap water.

TEP measurements were made the next day using 3 M KCl-agar bridges connected via Ag/AgCl electrodes (World Precision Instruments, Sarasota, FL, USA) to a Radiometer pHM 82 pH meter (Radiometer, Copenhagen, Denmark), which served as a high impedance voltmeter. The measurement bridge was connected to the saline-filled intraperitoneal catheter, and the reference bridge was placed in the water of the measurement chamber. TEP measurements in each experimental solution were made in triplicate over a 2-min period, then averaged, with correction for the junction potential which was recorded both before and after each measurement.

For each experimental series, the measurement bridge was connected to the catheter while the fish rested in control background water, and then the fish was transferred to the measurement chamber which was also filled with fresh background water. The TEP measured in this water served as the baseline TEP against which all subsequent experimental values were compared in that fish. For subsequent determinations, the fish was briefly returned to background water for 1 min as a rinsing procedure, and then transferred to the new experimental treatment. TEP values generally stabilized within 2–3 min in each new experimental solution. As 9 treatments (control background plus 8 salt levels in increasing concentrations) were employed in each series, the time needed to complete a series for one fish was about 40–50 min. Test waters were renewed after each fish. As outlined in Results, responses to salt exposures were expressed as changes in TEP (i.e., ΔTEP relative to baseline). Each series comprised 6–7 fish per species. In general, each fish was used for 2–4 experimental series with a recovery period of > 3 h in between.

Two additional series were performed to address specific objectives. In one series, cannulated representatives of all three species were exposed to a 900 mmol L⁻¹ solution of mannitol in Miami tap water for 3 min. Mannitol is an inert sugar which exerts osmotic pressure but carries no ionic charge. The goal was to evaluate whether any of the TEP responses could have occurred in response to elevated osmolarity. The TEP was first measured in background water (baseline TEP) and then after acute exposure to mannitol (900 mmol L⁻¹). At this concentration, it exerts an osmotic pressure slightly greater than that of the highest salt concentration tested in our studies (436 mmol L⁻¹ NaCl). In the other series, cannulated CC, which proved to be the most robust of the three species in their tolerance of repeated handling, were exposed to the 5th concentration (i.e. 50 % of the LC50) of each of the 8 salts for 24 h. Different animals were used for each salt test, with repeated measurements on the same animals first in background water (baseline TEP), then after acute transfer at 0 h, and at 4 h and 24 h of continuous exposure to the salt. After the 0-h measurement, the fish were returned to a common 40-L tank served with aeration and containing the appropriate concentration of the salt of interest, to ensure that all fish were exposed to the same condition. The catheters were labeled for identification of the individuals, and the fish were briefly transferred to the 200-mL measurement chambers for TEP recording at 4 h and 24 h. A control group was treated similarly but exposed to background Miami tap water throughout. Note that within a treatment, the same individual animals were followed over time, and all ΔTEP responses were expressed relative to the pre-treatment baseline TEP values measured in background water in the same fish.

After completion of all experiments, fish were euthanized with an overdose of neutralized MS-222 (1 g L⁻¹) and weighed.

2.4. Statistics

Data have been expressed as means \pm 1 SEM (N = number of fish). Linear and non-linear regressions were performed in GraphPad Prism, Version 8 (GraphPad Software, San Diego, CA, U.S.A.), and the significance of R² values assessed. Non-linear regression was used to fit concentration kinetic curves to experimental TEP data, using the Michaelis-Menten equation. This equation is often used to describe substrate concentration versus velocity relationships for enzyme or

transport kinetics:

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

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

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

$[\text{Salt}]_o$ = external salt concentration (mmol L⁻¹)

K_m = affinity constant (mmol L⁻¹) representing the $[\text{Salt}]_o$ associated with 50 % of $\Delta\text{TEP}_{\text{max}}$

Two-way ANOVA (factors: species, salt treatment) followed by Tukey's test for multiple comparisons, or one-way repeated measures ANOVA followed by Dunnett's test (to identify significant differences over time from the control) were employed. Student's two-tailed one-sample *t*-test was used to determine whether responses were significantly different from zero. Where necessary, data were appropriately transformed to ensure normality and homogeneity of variances. A significance level of *P* < 0.05 was used throughout.

3. Results

Within each species, baseline TEP values in Miami tap water were generally below 0 mV (i.e. negative) expressed as the inside voltage relative to the outside water. They were also rather variable (FHM: -12.0 to -1.0 mV; CC: -14.0 to +1.3 mV; BG: -7.3 to +2.7 mV). Values were also variable among species, with FHM [-6.8 \pm 0.6 (n = 35) mV] and CC [-5.3 \pm 0.5 (n = 77) mV] exhibiting similar mean baseline values that were both significantly more negative than those of BG [-2.9 \pm 0.6 mV (n = 22)]. There were no significant relationships between baseline TEP values and body weight in any of the species (R² = 0.01 – 0.04). Baseline TEP values were repeatable over time in individual animals and seemed to be characteristic of the individual; there were no significant differences in baseline TEP values (repeated measures analyses) when the same fish were used in several experimental series.

Changes in TEP (i.e., ΔTEP relative to baseline) in response to salt exposure were far more uniform than the absolute TEP values, reflecting the differences in baseline TEP, so all responses have been expressed as ΔTEP (e.g. Fig. 1), thereby greatly reducing variability. When fish were exposed to increasing concentrations of any of the eight salts, the TEP consistently increased above baseline in all three species, apart from the KCl series with BG where none of the ΔTEP responses were significantly different from zero (Fig. 1E). Note that in Fig. 1, the ranges of salt concentrations tested varied substantially amongst the different panels [e.g. NaCl up to 436 mmol L⁻¹ (Fig. 1A) but K₂SO₄ up to only 20 mmol L⁻¹ (Fig. 1F)], reflecting the vastly different toxicities of the various salts. Nevertheless, almost universally, the relationships of ΔTEP as a function of salt concentration were hyperbolic, approaching a plateau level at or before the 6th salt level, which represented the 96-h LC50 for FHM (Fig. 1).

These relationships between salt concentrations and ΔTEP were well-described by the Michaelis-Menten relationship (Eq. 2). Table 1 provides a summary of the $\Delta\text{TEP}_{\text{max}}$ and K_m values. Over the whole concentration range, ΔTEP responses were generally greatest in FHM (occasionally equal in CC) and least in BG. The overall differences between FHM and CC were significant for NaCl (Fig. 1A), Na₂SO₄ (Fig. 1B), KCl (Fig. 1E), and K₂SO₄ (Fig. 1F). For all salts, FHM exhibited significantly greater responses than BG (Fig. 1). Similarly, CC exhibited significantly greater responses than BG for all salts except NaCl (Fig. 1A). This pattern was also reflected in the $\Delta\text{TEP}_{\text{max}}$ values, but not in the K_m values where the only significant differences were the lower value for NaCl in CC relative to the other two species, and the higher value for MgCl₂ in BG relative to the other two species (Table 1). In Fig. 2, these relationships of ΔTEP response versus salt concentration have been compared within FHM when the salt exposures are expressed in four different ways – as % of FHM 96-h LC50 (Fig. 2A), as

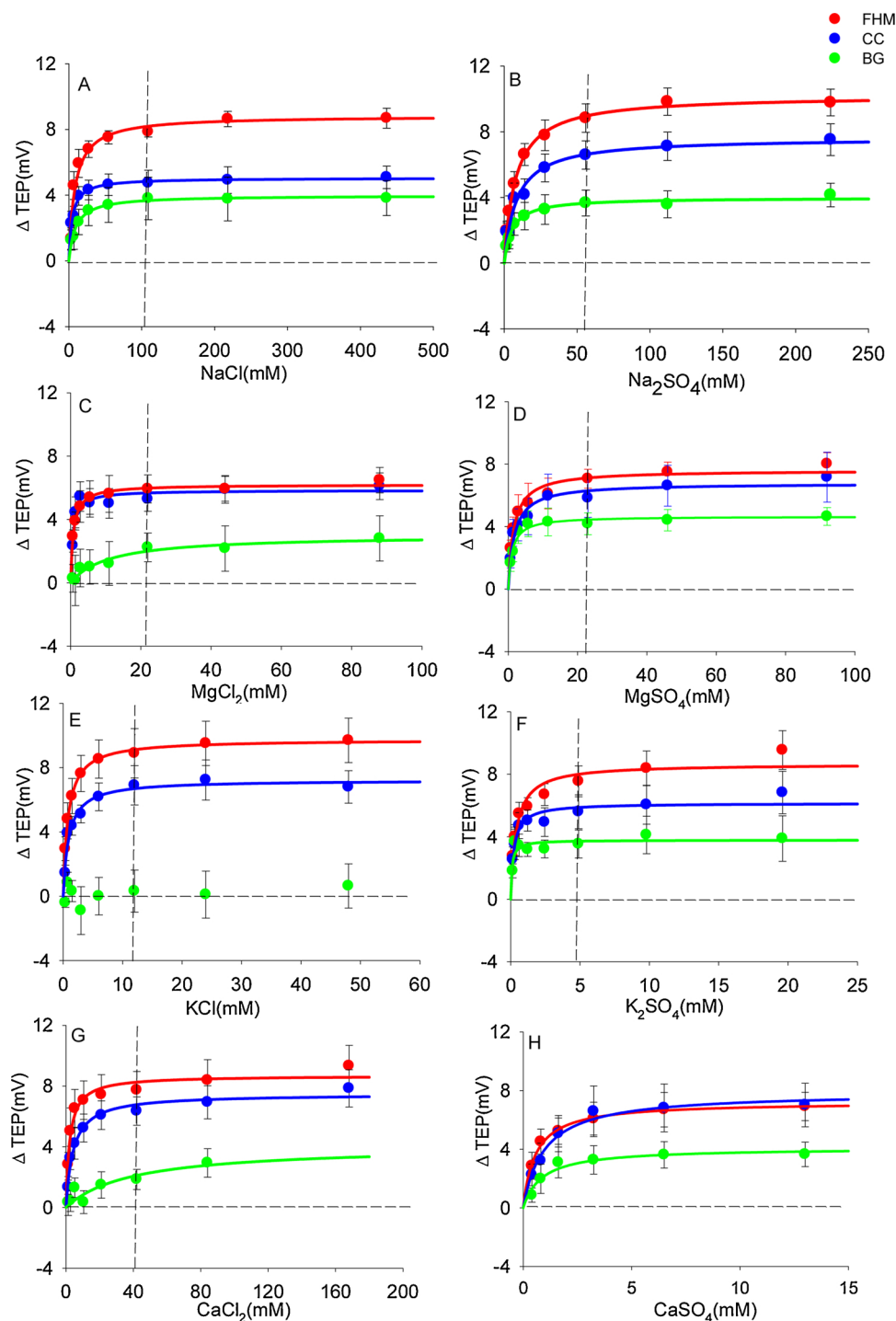


Fig. 1. Changes in TEP (i.e. Δ TEP relative to baseline, in mV) in fathead minnow (FHM red symbols), channel catfish (CC, blue symbols), and bluegills (BG, green symbols) in response to acute salt exposure, in mmol L^{-1} (A) NaCl; (B) Na_2SO_4 ; (C) MgCl_2 ; (D) MgSO_4 ; (E) KCl; (F) K_2SO_4 ; (G) CaCl_2 ; and (H) CaSO_4 . A geometric concentration series was tested representing 3.12 %, 6.25 %, 12.5 %, 25 %, 50 %, 100 %, 200 %, and 400 % of the 96-h LC50 value for juvenile FHM (from Mount et al., 1997), with the dashed vertical line representing 100 % of the LC50 for FHM, except in panel H where the highest concentration (the 6th solution) is slightly below the solubility limit for CaSO_4 , so no LC50 is shown. Means \pm 1 SEM (N = 6-7). The lines fitted to the means are given by the Michaelis Menten equation. See Table 1 for constants and fitting parameters. By two-way ANOVA, the relationships were significantly different from one another among all three species in Panels A, B, E, and F. In panels C, D, G and H, FHM and CC relationships were not significantly different, but both were significantly different from the BG relationships.

concentration in mmol L^{-1} (Fig. 2B), as TDS in mg L^{-1} (Fig. 2C), and as conductivity in $\mu\text{S cm}^{-1}$ (Fig. 2D). Clearly, variability in the Δ TEP responses at a given exposure over the whole concentration-response curve is greatly reduced when the exposure concentration is expressed as % of 96-h LC50 (Fig. 2A), in contrast to the other units sometimes used in environmental regulations (Figs. 2B,C,D). Supplementary Figs. S1 and S2 provide comparable analyses for CC and BG, and lead to the same conclusion that variability is greatly reduced for these species as well, when the exposure concentration is expressed as % of 96-h LC50, rather than other commonly used units. Note however that there was greater dispersion in the BG responses.

Of particular interest is the fact that regardless of which salt was tested, the relationships reached a plateau level at or below the LC50

concentration for FHM, as marked with dashed lines in Fig. 1. For CaSO_4 (Fig. 1H), no LC50 concentration is marked because the 6th concentration (in this case the final concentration tested) was close to the solubility limit, yet the LC50 for FHM had still not been reached. When the mean Δ TEP values at the LC50 concentrations for the eight different salts were compared with one another (for CaSO_4 , the plateau value at the highest concentration tested was used instead), there was remarkable uniformity within a species, though differences in absolute magnitude among the species persisted (FHM \geq CC > BG). The uniformity is illustrated for FHM in Fig. 3A, where overall variation in mean values of this metric (Δ TEP value at the LC50) was at most only about 1.5-fold (i.e. + 5.9 mV for MgCl_2 versus + 8.9 mV for KCl). The same situation was true for CC where mean Δ TEP at the LC50 varied

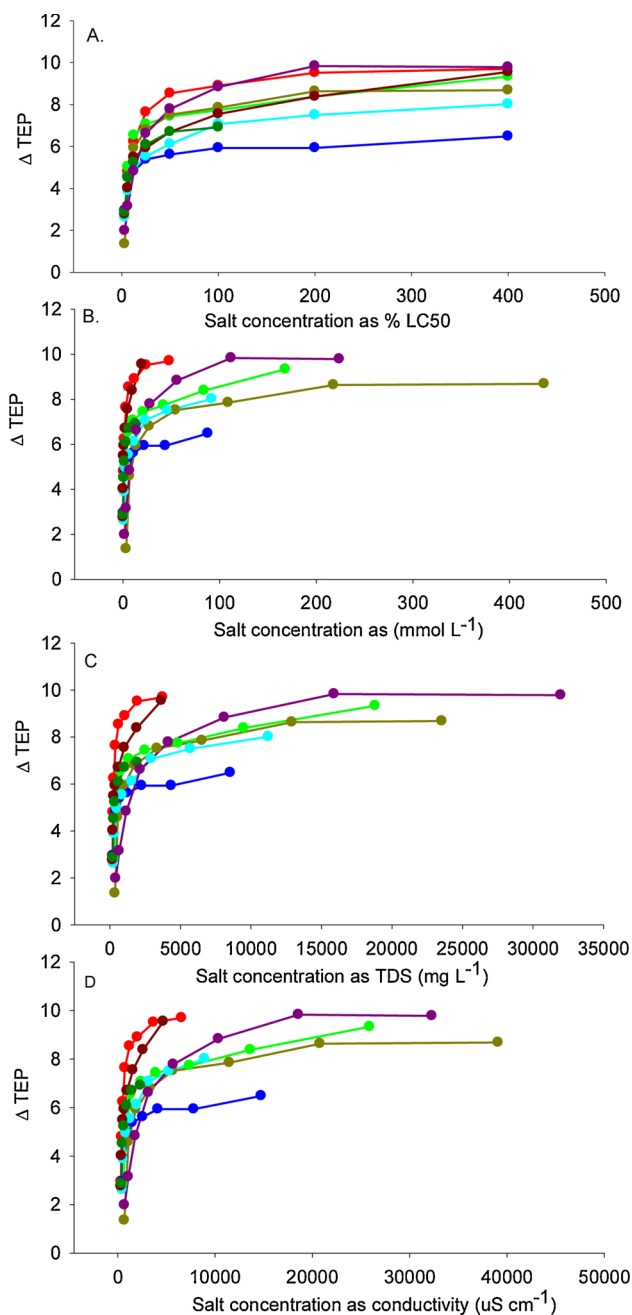


Fig. 2. Changes in TEP (i.e. Δ TEP relative to baseline, in mV) in fathead minnow (FHM) in response to eight different salts, with the salt concentrations expressed on a common scale in four different ways: (A) as percent of the 96-h LC50 for juvenile FHM (from Mount et al., 1997), with the same caveat for CaSO_4 as in the legend of Fig. 1; (B) as mmol L^{-1} ; (C) as total dissolved solids (TDS) in mg L^{-1} ; and (D) as conductivity in $\mu\text{S cm}^{-1}$. Note the much greater dispersion of the data in panels B, C, and D, relative to panel A. Each salt is plotted with a different symbol as noted on the Figure. Only means ($N = 6-7$) are plotted; SEMs (identical to those for FHM in Fig. 1) have been removed for clarity.

1.4-fold from +4.8 mV for NaCl to +6.9 mV for CaSO_4 . For the same analysis in BG (Fig. 3C), we excluded the KCl value (Fig. 1E) because it was not significantly different from zero. The other seven Δ TEP responses at the LC50 varied 2.2-fold from +1.9 mV for CaCl_2 to +4.2 mV for MgSO_4 . This very low 1.4- to 2.2-fold variation in the Δ TEP responses at the LC50 was also reflected in the Δ TEP_{max} values calculated by Michaelis-Menten analyses where variation was only 1.3- to 1.7-fold within species (Table 1). This uniformity may be contrasted with 28-fold variation in the salt concentration when expressed on a molar basis (3.9 mmol L^{-1} for K_2SO_4 to 109 mmol L^{-1} for NaCl; Fig. 2B, Supplementary Figs. S1B, S2B), 9.6-fold variation when expressed as TDS (849 mg L^{-1} for K_2SO_4 to 8123 mg L^{-1} for Na_2SO_4 ; Figs. 2C, S1C, S2C), and 7.3-fold variation when expressed as conductivity ($1563 \mu\text{S cm}^{-1}$ for K_2SO_4 to $11,480 \mu\text{S cm}^{-1}$ for NaCl; Figs. 2D, S1D, S2D).

While the Δ TEP_{max} values were uniform amongst different salts, this was not true for the K_m values which define the position of the

concentration-kinetic curves (Table 1). At least for FHM and CC, the highest affinities (i.e. lowest K_m values) were seen for the potassium salts, and the lowest affinities (i.e. highest K_m values) for the sodium salts, with the calcium and magnesium salts exhibiting intermediate values (Table 1). In Fig. 4, log K_m values (Y-axis) of the different salts (Table 1) were regressed against log 96-h LC50 values (X-axis) for the same salts in FHM, plus four values for 96-h LC50 in BG and two for 96-h LC50 in CC, from sources described in Section 2.2 of Methods. There was a strong positive relationship ($R^2 = 0.859$, $P < 0.00001$, $N = 13$).

For FHM, plots of the Δ TEP data separately against the concentrations of common cations (Na^+ or K^+ or Mg^{2+}) or the common anions (Cl^- or SO_4^{2-}) confirmed that response patterns were largely driven by the cations rather than by the anions. For example, for sodium salts, the plateau was reached at 96-h LC50 values ranging from 109.3 to 112.0 $\text{mmol L}^{-1} \text{Na}^+$ (Fig. 5A), for magnesium salts the plateau was reached at LC50 values ranging from 22.3 to 23.4 $\text{mmol L}^{-1} \text{Mg}^{2+}$ (Fig. 5B), and for potassium salts the plateau Δ TEP was reached at LC50 values

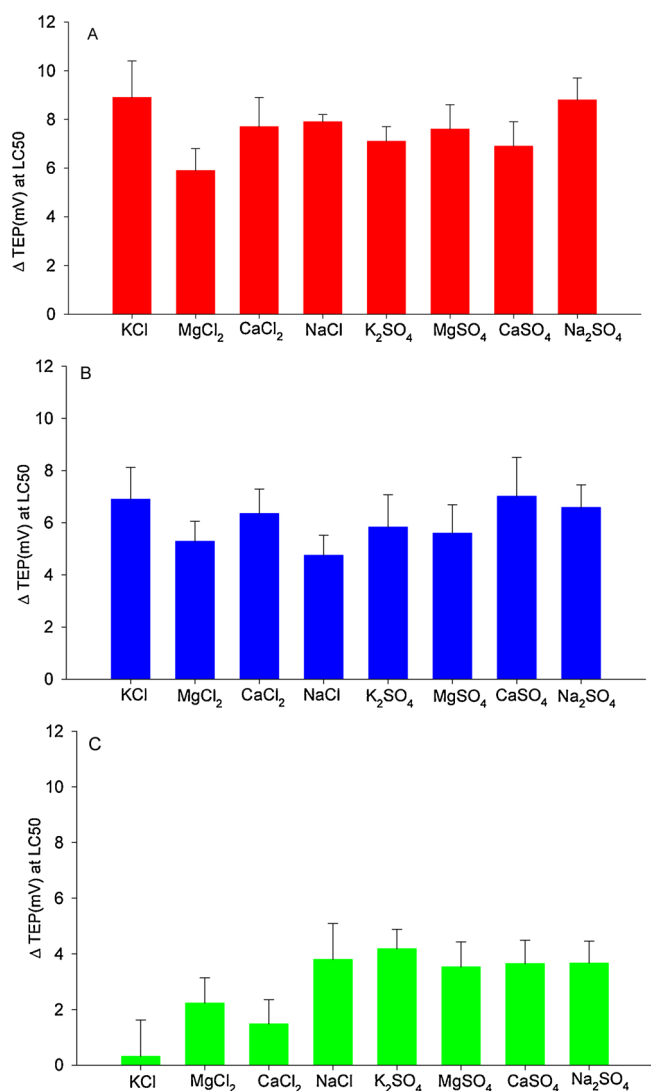


Fig. 3. The responses in TEP (i.e. ΔTEP relative to baseline, in mV) at the 96-h LC50 concentrations for eight different salts in (A) fathead minnow (FHM); (B) channel catfish (CC); and (C) bluegill (BG). Means \pm 1 SEM (N = 6-7). For CaSO₄, the response at the plateau has been plotted, reflecting the caveat noted in the legend of Fig. 1. Note that within a species, there were no significant differences among the responses to the eight salts. However, by two-way ANOVA, there were overall significant differences among the three species, with FHM responses being greater than CC responses which were greater than BG responses.

ranging from 7.8 to 11.8 mmol L⁻¹ K⁺ (Fig. 5C). In contrast, for chloride salts the plateau ΔTEP was reached at LC50 values varying from 11.8 to 109.3 mmol L⁻¹ Cl⁻ (Fig. 5D) and for sulphate salts from 3.9 to 56.0 mmol L⁻¹ SO₄²⁻ (Fig. 5E). Note that we did not include calcium salts in this analysis because of the solubility issue with CaSO₄. Comparable plots for CC (Supplementary Fig. S3) were less definitive but supported a similar conclusion. However, for BG, this difference was not readily apparent, with convergence for some responses (e.g., Na⁺, Supplementary Fig. S4A) but not others (e.g., Mg²⁺, K⁺, Supplementary Figs. S4B, C) as a function of the cation concentration. Convergence of responses as a function of the anion concentration (e.g., Cl⁻, SO₄²⁻, Supplementary Figs. S4D, E) was perhaps marginally better for BG.

Exposure to 900 mmol L⁻¹ mannitol caused no change in TEP in any of the three species (Fig. 6), showing that none of the ΔTEP responses could be caused by elevated osmolarity. In CC, we followed the ΔTEP responses over 24 h of continuous exposure to 50 % of the LC50

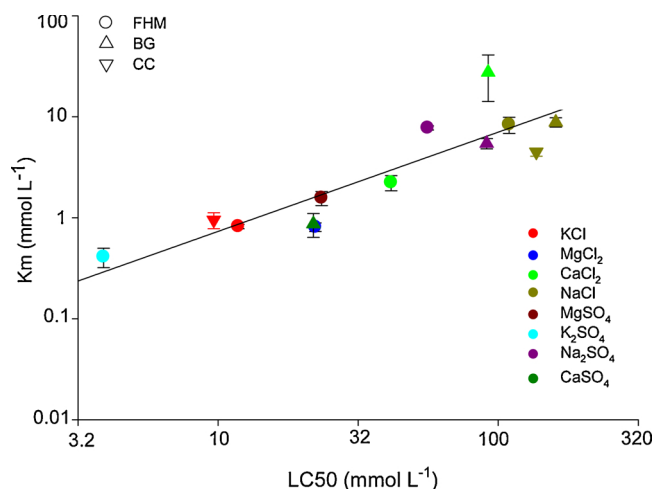


Fig. 4. The linear regression relationship between log Km values [Y, mmol L⁻¹, affinity constant for ΔTEP (means \pm 1 SEM, N = 6, from Table 1)] for 7 different salts in fathead minnow (FHM) versus log 96-h LC50 values (X, mmol L⁻¹) for the same 7 salts in FHM from Mount et al. (1997), plus 4 different salts for bluegill (BG) and 2 different salts for CC. The BG and CC 96-h LC50 values were obtained as geometric means from USEPA (1988) and from the US EPA ECOTOX data base (<https://cfpub.epa.gov/ecotox/>). The equation is $Y(\log Km) = 0.9829X(\log LC50) - 1.1179$ ($R^2 = 0.859$, $P < 0.00001$, $N = 13$).

concentrations of each of the eight different salts, together with the responses of a procedural control group that were kept in background water throughout (Fig. 7). There were no significant changes over time (repeated measures ANOVA) in ΔTEP responses in any of the eight salt exposures or in the control treatment (Fig. 7).

4. Discussion

4.1. Support for the assumptions of the MIT model

The first four of our original objectives (see Introduction) addressed assumptions of the MIT model (EPRI, 2018). We found that (i) all salts tested disturbed the TEP across the gills by driving it to less negative or more positive values (the same direction of change as predicted by the MIT model), but that the concentration-response relationships varied greatly depending on the toxicities of the different salts (Figs. 1, 2, and S1 and S2). The responsiveness of TEP scaled with the individual salt toxicities, exactly as predicted by the MIT model. Therefore (ii) these concentration-response relationships became very consistent when concentration was expressed as a percentage of the 96-h LC50 for FHM, in accord with the model. This conclusion also applied to CC and BG even though the LC50 data were taken from FHM, reflecting the fact that the 96-h LC50 values for major salts do not vary greatly amongst fish species. The additional 96-h LC50 values for BG (4 values) and CC (2 values) included in Fig. 4 differed at most by only 1.5- and 2.2-fold respectively from the FHM values. Furthermore (iii) a consistent acute disturbance (i.e. ΔTEP relative to baseline) was predictive of 96-h mortality regardless of the salt causing it. This turned out to be the ΔTEP value associated with the 96-h LC50, which varied at most by 1.4 to 2.2-fold within a species. Finally, we confirmed (iv) that this ΔTEP at the LC50 concentration was much less variable than the salt exposures causing it, whether these were expressed as the molar concentrations, TDS values, or conductivity values. In total, these empirical findings provide strong physiological support for the basic assumptions of the MIT model, which prior to this was an entirely theoretical exercise based on toxicity data and assumptions about permeability values and extracellular fluid ion concentrations (EPRI, 2018, and previous versions referenced therein).

We suggest that in future it may be possible to use the MIT model in

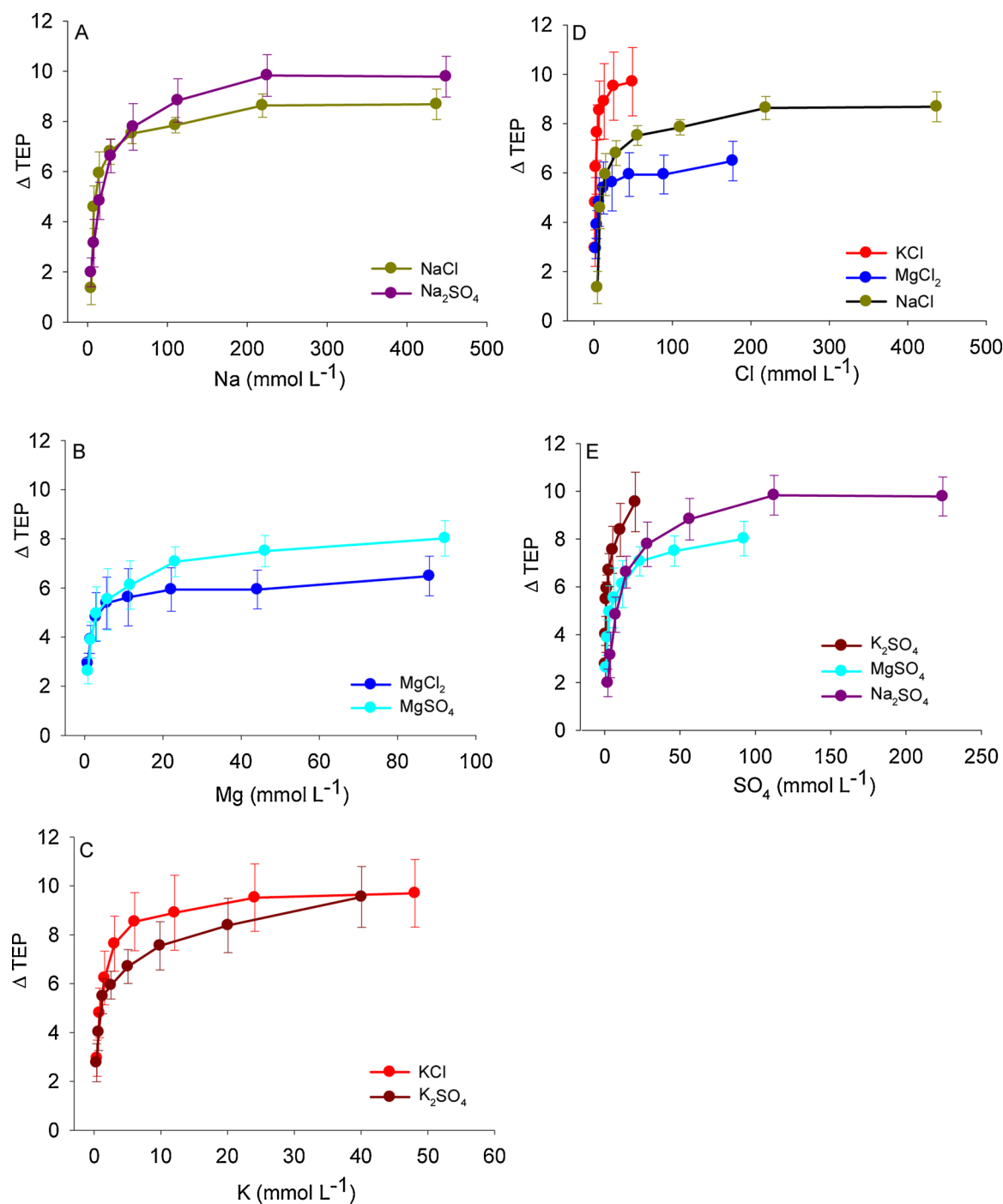


Fig. 5. The responses in TEP (i.e. ΔTEP relative to baseline, in mV) in fathead minnow (FHM) to six different salts plotted as a function of the common cation concentration, in mmol L⁻¹ [left hand panels (A) Na⁺; (B) Mg²⁺; and (C) K⁺] or for the same salts plotted as a function of the common anion concentration, in mmol L⁻¹ [right hand panels (D) Cl⁻ and (E) SO₄²⁻]. Means ± 1 SEM (N = 6–7). Note the convergence of response patterns as a function of the common cation concentration, and the wide dispersion of response patterns as a function of the common anion concentration.

an analogous manner as the Biotic Ligand Model (BLM) that is now widely used for risk assessment of metal toxicity (Di Toro et al., 2001; Paquin et al., 2002; Niyogi and Wood, 2004; Mebane et al., 2020). The BLM is based on knowledge that the metal burden on the gills within a species is much less variable than the metal levels in different exposure waters that cause it, and is an excellent predictor of ultimate mortality. The BLM uses geochemical modeling to predict the short-term metal burden on the gills, and in turn the ultimate toxicity that is associated with this burden. Differences in sensitivity among species are dealt with by appropriate adjustments of the LA50 (sensitivity factor = lethal gill

metal accumulation associated with 50 % mortality at a given time). Similarly, the ΔTEP at the gills within a species is much less variable than the salt levels in the exposure waters that cause it, and again is an excellent predictor of ultimate mortality. Given this consistency and the strong relationship between log *K_m* and log 96-h LC50 (Fig. 4), electrochemical modeling could be used to predict the short-term ΔTEP value at the gills, and in turn the associated toxicity. Differences in sensitivity among species could be dealt with by appropriate adjustments of the ΔTEP50 (sensitivity factor = disturbance in TEP associated with 50 % mortality at a given time). As the plateau ΔTEP50 for

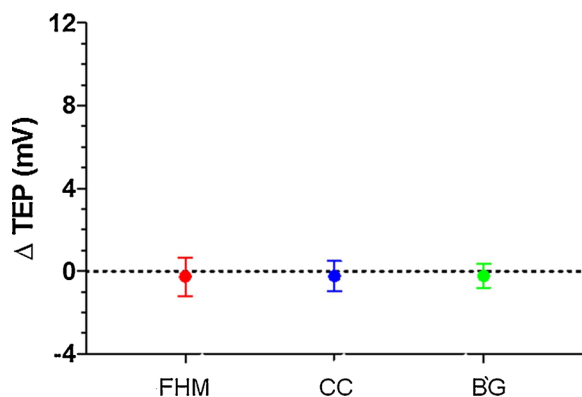


Fig. 6. The lack of response in TEP (i.e. Δ TEP relative to baseline, in mV) in fathead minnow (FHM), channel catfish (CC), and bluegill (BG) to acute exposure to 900 mmol L⁻¹ mannitol, an inert sugar chosen to exert an osmotic pressure slightly greater than that of the highest salt concentration tested in the present studies (436 mmol L⁻¹ NaCl). Means \pm 1 SEM (N = 6). None of the responses were significantly different from zero, or from each other.

some salts appears to be reached below the 96-h LC50, the model would err on the side of conservatism.

4.2. Why does salt exposure cause increases in TEP and why are these associated with toxicity?

The findings associated with the final three of our original objectives help cast light on these issues. We found that (v) Δ TEP responses were more strongly associated with the cationic components rather than the anionic components of the salts in two of the three species (FHM and CC), and this was directly related to toxicity (Figs. 5; S3). Highly toxic K⁺ salts with low LC50 values (KCl and K₂SO₄) caused large Δ TEP responses at low concentrations, moderately toxic Mg²⁺ salts (MgCl₂ and MgSO₄) did the same at moderate concentrations, and fairly benign salts with high LC50 values (NaCl and Na₂SO₄) exerted quantitatively similar Δ TEP effects only at high concentrations. These differences were relatively uninfluenced by the accompanying anion (Cl⁻ or SO₄²⁻). These findings agree with the cationic diffusion potential model for gill TEP (see below). While both environmental regulations and earlier research work focus on anions (see Introduction, Mount et al., 1997), recent studies have more strongly emphasized the roles of cations in causing salt toxicity (Mount et al., 2016; Erickson et al., 2017, 2018). Notably, this dependence on the cation rather than the anion was not apparent in the BG (Supplementary Fig. S4), which

may be a special case (Section 4.3).

With respect to objective (vi), the high mannitol exposure trials demonstrated a complete lack of effect of osmolality on TEP (Fig. 6), in agreement with other TEP studies on fish (Potts and Eddy, 1973; Wood and Grosell, 2008). Whether osmolality contributes to salt toxicity in fish remains an open question, though it does contribute to the toxicity of Na⁺-dominated solutions in *Ceriodaphnia dubia*, a species for which hemolymph osmolality is much lower than that in freshwater teleosts (Mount et al., 2016; Erickson et al., 2017). Finally, for objective (vii), we found that sublethal Δ TEP responses to all the salts were stable over time, at least up to 24 h (Fig. 7), which suggests that fish are not able to compensate for these TEP disturbances over this time period.

Classic investigations dating back 40–50 years (Kerstetter et al., 1970; Potts and Eddy, 1973; Eddy, 1975; McWilliams and Potts, 1978; Potts, 1984) have established our current understanding of TEP in freshwater fish. In brief the TEP, which is almost always negative, is largely or entirely a diffusion potential resulting from the differential permeability of the gill epithelium to cations *versus* anions ($P_{cation} > P_{anion}$). As the major extracellular ions are Na⁺ and Cl⁻, the combination of the extracellular $[Na^+]_i > [Cl^-]_i$ and $P_{Na} > P_{Cl}$ (see Eq. 1) results in the inside negative TEP. Several authors have suggested that there may also be an electrogenic component to the TEP in freshwater fish (Eddy, 1975; Potts, 1984). There is little evidence for this, but Eddy (1975) noted that an electrogenic component would likely display Michaelis-Menten kinetics when substrate (i.e. $[ion]_o$) was increased. Given that virtually all of our data were well-described by the Michaelis-Menten equation (Fig. 1, Table 1), we became concerned this possibility might apply. A simple test to eliminate this possibility is to measure the TEP immediately after transfer of the fish to physiological saline (Potts, 1984). All diffusion gradients would be eliminated, but an electrogenic component associated with active transport would continue for some time. During the data analysis phase of our study, we had the opportunity to test this with FHM only, because the CC and BG were no longer available. The results clearly showed the absence of an electrogenic component, because the TEP immediately rose from approximately -11 mV in baseline water to a value not significantly different from 0 mV after acute transfer of FHM to Cortland saline (Wolf, 1963) (Fig. 8). Subsequent studies on two other species, rainbow trout and goldfish, yielded a similar result (B.H.K. Po and C.M. Wood, unpubl. data). We are therefore reasonably confident that only diffusion potentials are present.

Thus, a simple explanation of the progressively more positive TEP caused by increasing concentrations of all the salts is that the increased inward diffusion gradient of cations, or in the case of Na⁺, the reduced outward diffusion gradient for Na⁺, drives the TEP progressively more

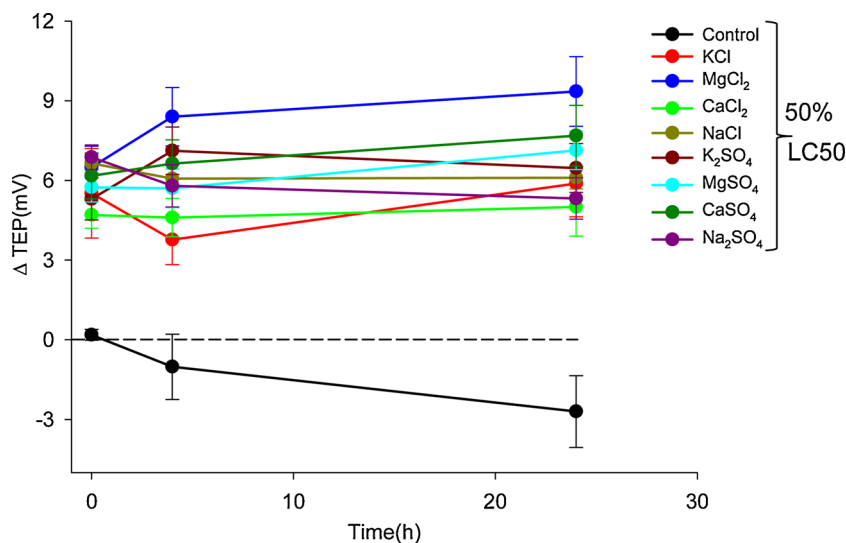


Fig. 7. A test of the stability over time, using channel catfish (CC), of the responses in TEP (i.e. Δ TEP relative to baseline, in mV) in fish exposed to the 5th concentration (i.e. 50 % of the LC50 for FHM) of each of the eight salts for 24 h. Different animals were used for each salt test, with repeated measurements on the same animals first in background water (baseline TEP), then after acute transfer at 0 h, and at 4 h and 24 h of continuous exposure to the salt. All Δ TEP responses were expressed relative to the pre-treatment baseline TEP values measured in background water in the same fish. A control group was treated similarly but exposed to background Miami tap water throughout. Means \pm 1 SEM (N = 6). By repeated measures ANOVA, there were no significant differences over time relative to the mean Δ TEP in that treatment measured at 0 h (Dunnnett's test). By two-way ANOVA, there were also no significant differences among the responses to the eight salt treatments, but all were significantly different from the control responses.

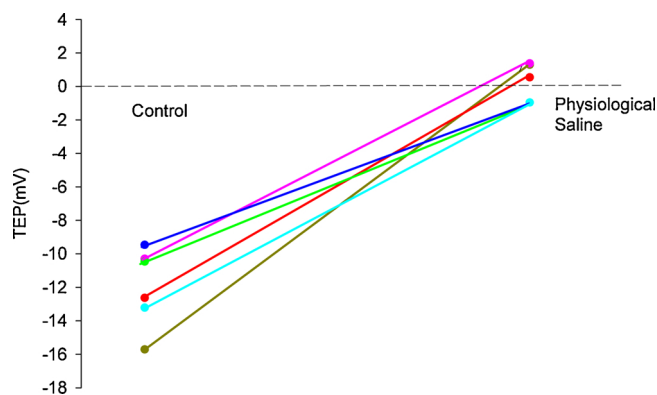


Fig. 8. A test in fathead minnow (FHM) showing the absence of an electrogenic component to the TEP. Immediately after acute transfer of FHM from Miami tap water (baseline condition) to Cortland saline (Wolf, 1963), the negative TEP rose to a value not significantly different from 0 mV. Each line represents one fish (N = 6).

positive. This would explain why the effect is closely correlated with the specific cation concentration, and not with the specific anion concentration, at least for FHM and CC, in accord with recent toxicity interpretations (Mount et al., 2016; Erickson et al., 2017, 2018). The differential potency of the various cations (e.g. $K^+ > Mg^{2+} > Na^+$) might therefore simply reflect differential permeability. Indeed, there is abundant evidence that K^+ is more permeable than Na^+ across the fish branchial epithelium (Potts, 1984). Michaelis-Menten saturation kinetics could reflect either homeostatic permeability adjustments and/or saturation of channels in the gills.

Ca^{2+} may represent a special case. In the present data set, a plateau in ΔTEP was reached in the standard fashion just below the LC50 for $CaCl_2$ (Fig. 1G), but for $CaSO_4$, the plateau occurred at a much lower concentration (Fig. 1H), even though there was no LC50 due to the solubility limit being reached first. Nevertheless, Michaelis-Menten relationships occurred for both salts. Several previous authors have increased $[Ca]_o$ and found similar progressive increases in ΔTEP which eventually reach a plateau in a Michaelis-Menten fashion (Eddy, 1975; McWilliams and Potts, 1978; Wood et al., 1998). While the same explanation as for other cations may apply in part (i.e. an increased gradient for the diffusive influx of Ca^{2+}), there is abundant evidence [reviewed by Potts (1984) and Evans et al. (2005)] that external Ca^{2+} also serves to reduce the permeability of fish gills to many substances, including ions. Ca^{2+} helps tighten the paracellular junctions between the gill cells, and by titrating the negative charge on these pathways, it changes their permselectivity. While they become less permeable overall, their relative anion-to-cation permeability increases, both of which will make the TEP more positive. Further complicating the situation is evidence that higher water Ca^{2+} levels may either mitigate or exacerbate major salt toxicity (e.g. Davies and Hall, 2007; Elphick et al., 2011a, b; Mount et al., 2016; Erickson et al., 2017, 2018; Bogart et al., 2019), so in the future, it will be of interest to test whether ΔTEP responses to various cations are similarly affected.

Finally, we are left with the question of why salt-induced increases in ΔTEP close to the plateau level correspond so well with salt concentrations causing eventual mortality. Are the increases in ΔTEP a cause of toxicity, or merely a symptom? At present we cannot answer that question, but it is interesting that ΔTEP disturbances persist without correction over time (Fig. 7). Theoretically, a more positive TEP would impede net Na^+ uptake at the gill while aiding net Cl^- uptake. Plasma $[Na^+]_i$ would be predicted to slowly fall and plasma $[Cl^-]_i$ would be predicted to slowly rise. By Strong Ion Difference Theory (Stewart, 1978, 1983), an ensuing metabolic acidosis should occur. Either the ionic disturbance alone, and/or the acidosis could be the eventual cause of death.

4.3. Why does the bluegill (BG) respond differently than the fathead minnow (FHM) and channel catfish (CC)?

We chose the bluegill for study as it is one of only a handful of freshwater fish discovered to date to have a fundamentally different ionoregulatory system, lacking active Cl^- uptake at the gills, so that all Cl^- must be obtained from the diet (Tomasso and Grosell, 2005). The common killifish (mummichog), *Fundulus heteroclitus*, a euryhaline teleost, shares this trait when acclimated to freshwater (Patrick et al., 1997; Wood and Laurent, 2003; Bucking et al., 2013) and its TEP has been studied (Wood and Grosell, 2008). The killifish exhibits some remarkable similarities to the BG, including (a) a baseline TEP in Miami tap water which is very close to 0 mV; (b) a complete insensitivity of the TEP to $[K^+]_o$, and (c) a comparably low sensitivity of the TEP to $[Na^+]_o$. It differs however in exhibiting a complete insensitivity of the TEP to both $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ whereas the BG is moderately responsive to these ions. It is possible that in both species, the diffusion channels in the gills are virtually closed to ensure maximum retention of Cl^- , so that external cations have very little effect on TEP.

4.4. Future directions

While the present study has provided strong support for the assumptions of the MIT model (EPRI, 2018), it has also highlighted some important targets for future research. (1) In light of the deviations of the BG data from some of the patterns seen in FHM and CC, there is a need to test a wider range of species with different ionoregulatory mechanisms. Daphnids will be particularly important because they appear to be much more sensitive to salt toxicity than fish (Mount et al., 1997; Tietge et al., 1997), they have very different ion transporters (Glover and Wood, 2005; Bianchini and Wood, 2008), and they have been the focus of extensive modeling efforts recently (Mount et al., 2016; Erickson et al., 2017, 2018; EPRI, 2018). Further, it is necessary to test the MIT model for other invertebrates such as mollusks (e.g. Gillis, 2011; Cañedo-Argüelles et al., 2016; Wang et al., 2018; Bogart et al., 2019) and mayflies (Kunz et al., 2013; Soucek and Dickinson, 2015; Soucek et al., 2018), both of which appear highly sensitive to major ion contamination, and are in general decline across North America. (2) As we do not yet know how or why a certain positive ΔTEP is associated with toxicity, experiments in which the blood is sampled sequentially for changes in ionic and acid-base status during salt exposure would be very informative to test our speculation that extracellular $[Na^+]_i$ declines, $[Cl^-]_i$ increases, and metabolic acidosis ensues. (3) An added benefit is that measurements of internal ion concentrations, together with external concentrations and TEP, will facilitate direct calculation of relative ion permeabilities. These can then replace the “fitted” values currently employed in modeling. (4) As water hardness, particularly $[Ca^{2+}]_o$, clearly alters multi-ion toxicity, we need to understand how acclimation of organisms to different calcium concentrations affects their ΔTEP responses to different salts. (5) And most importantly, the ultimate goal of the modeling efforts is to predict the responses not to just single salts, but rather to salt mixtures that commonly occur in contaminated environments. It will be important to understand how to use the ΔTEP metric (validated for single salts here) to predict the response to salt mixtures.

Author contributions

CMW and KVB conceived the project, performed most of the experiments, and analyzed the data; BHKP performed additional experiments; MG, MDM, WJM, and DRM provided resources and advice; CMW wrote the first draft of the MS; all authors revised the MS.

Declaration of Competing Interest

We declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.aquatox.2020.105568>.

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