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Relative Toxicity and Sublethal Effects of NaCl and Energy-Related Saline Wastewaters on Prairie Amphibians

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ABSTRACT

Increasing salinity in freshwater environments is a growing problem due both to the negative influences of salts on ecosystems and their accumulation and persistence in environments. Two major sources of increased salinity from sodium chloride salts (NaCl) are saline wastewaters co-produced during energy production (herein, wastewaters) and road salts. Effects of road salts have received more attention, but legacy contamination from wastewaters is widespread in some regions and spills still occur. Amphibians are sensitive to contaminants, including NaCl, because of their porous skin and osmoregulatory adaptations to freshwater. However, similarities and differences between effects of wastewaters and road salts have not been investigated. Therefore, we investigated the relative influence of wastewaters and NaCl at equivalent concentrations of chloride on three larval amphibian species that occur in areas with increased salinity. We determined acute toxicity and growth effects on Boreal Chorus Frogs (*Pseudacris maculata*), Northern Leopard Frogs (*Rana pipiens*), and Barred Tiger Salamanders (*Ambystoma mavortium*). We posited that wastewaters would have additive effects on amphibians compared to NaCl because wastewaters often have additional toxic heavy metals and other contaminants. For NaCl, toxicity was higher for frogs than the salamander. Toxicity of wastewaters was also similar between chorus and leopard frogs. Only chorus frog survival was lower when exposed to wastewater compared to NaCl. Mass and length of leopard and chorus frog larvae decreased with increasing salinity after only 96 hours of exposure but did not for tiger salamanders. Size of leopard frogs was lower when exposed to NaCl compared to wastewater. However, growth effects were similar between wastewater and NaCl for chorus frogs. Taken together, our results suggest that previous studies on effects of road salt could inform future studies and management of wastewater-contaminated ecosystems, and vice versa. Nevertheless, effects of road salts and wastewaters may be context-, species-, and trait-specific and require further investigations. The negative influence of salts on imperiled amphibians underscores the need to restore landscapes with increased salinity and reduce future salinization of freshwater ecosystems.

1. Introduction

Increased salinity from anthropogenic sources, also described as secondary salinization, is an emerging stressor on freshwater environments (Dugan et al., 2017). Salinity, specifically increased chloride (Cl-) from sodium chloride salts (NaCl), has consistently increased in North American lakes (Dugan et al., 2017) and rivers over the past 50 years (Kaushal et al., 2005), but all types of freshwater ecosystems are subject to salinization (Hintz and Relyea, 2019). Salinity is a contaminant of particular concern because of its potential to persist and accumulate in environments (Dugan et al., 2017; Hintz and Relyea, 2019).

Although the effects of road salts have received more attention, legacy and current contamination from domestic energy production are also potential hazards to freshwater ecosystems (Gleason and Tangen, 2014; Lauer et al., 2016; Maloney et al., 2017; Vengosh et al., 2014). Saline wastewaters (herein, wastewaters) are a common byproduct of oil and gas extraction—more than 3.9 trillion liters per year of wastewaters are produced in the United States alone—and historical disposal practices and accidental spills have contaminated freshwater ecosystems in many areas (Cozzarelli et al., 2017; Gleason and Tangen, 2014; Maloney et al., 2017; Rozell and Reaven, 2012; Wanty, 1997). Wastewaters can contain high concentrations of NaCl and mixtures of heavy metals (e.g.,

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lead, selenium, strontium, antimony, and vanadium) and other contaminants (e.g., volatile organic compounds, hydrocarbons, and radionuclides), dependent on their origin (Cozzarelli et al., 2017; Gleason and Tangen, 2014; Lauer et al., 2016; Maloney et al., 2017). Wastewaters are also used to reduce dust and deice roads in more than 13 states, which can then leach into surrounding ecosystems (Tasker et al., 2018). Despite historical and continued contamination, sparse information exists for the effects of wastewaters on wetland-associated species, especially freshwater vertebrates (Davis et al., 2010; Maloney et al., 2017; Souther et al., 2014). The limited studies to date determined that wastewaters can decrease macroinvertebrate diversity (Preston and Ray, 2017), amphibian survival and abundance (Hossack et al., 2017; Hossack et al., 2018), and survival of Fathead Minnows (*Pimephales promelas*; Cozzarelli et al., 2017).

Road salts ostensibly have similar influences as wastewaters on freshwater ecosystems (reviewed in Hintz and Relyea, 2019) and NaCl is the primary constituent of road salts and wastewaters. Previous research on the effects of road salts could therefore guide management of wastewater-contaminated ecosystems. However, similarities and differences between effects of wastewaters and road salts have not been directly investigated. Other contaminants common to wastewaters could have additive or synergistic effects with NaCl (Beal et al., 1987; Farag and Harper, 2014). For example, studies with Wood Frogs (*Rana sylvatica)* and Boreal Chorus Frogs (*Pseudacris maculata*) highlight the potential for interactive effects between Cl- and other contaminants because survival was lower than expected for exposure to Cl- alone (Hossack et al., 2017; Snodgrass et al., 2008).

Amphibians, particularly their early life history stages, are sensitive to increased salinity because of their iono- and osmoregulatory adaptations to freshwater environments. Larval amphibians are hyperosmotic relative to their environment (Shoemaker and Nagy, 1977; Ultsch et al., 1999), therefore they are constantly exchanging water and ions with their aquatic environment through their gills and permeable skin. Increased conductivity from salinity or other contaminants can disrupt ionic and osmotic homeostasis of aquatic species (e.g., Brooks and Mills, 2003; Sola et al., 1995; Wilson and Taylor, 1993). Because of their sensitivity, amphibians are ideal model organisms to understand the influences of increased salinity and wastewaters on aquatic vertebrates (Hopkins, 2007). However, our understanding of the effects of disparate sources of increased salinity on amphibians is still limited.

Determining tolerances and sublethal effects of salinity from disparate sources on amphibians may help predict population-level effects and changes in aquatic communities to guide future research and enhance conservation and remediation efforts. Therefore, our objective was to determine the relative influences of wastewaters and NaCl on survival and growth of larval amphibians in prairie wetlands from the Williston Basin, a large energy reserve in central North America where wastewater contamination is pervasive (Gleason and Tangen, 2014). We posited that wastewaters would have additive effects compared to NaCl because of additional contaminants found in wastewaters. However, if effects of wastewaters are predominantly due to NaCl, previous studies on effects of road salt could inform future studies and management efforts for ecosystems contaminated by wastewaters.

2. Methods

2.1. Study organisms, egg collection, and husbandry

We studied three amphibian species native to the Prairie Pothole Region (PPR) of north-central North America: Boreal Chorus Frogs (herein, chorus frogs; *Pseudacris maculata*), Northern Leopard Frogs (herein, leopard frogs; *Rana pipiens*), and Barred Tiger Salamanders (herein, tiger salamanders; *Ambystoma mavortium*). All three amphibian species are widely distributed in northern and western North America, where they are likely to experience salt contamination from different sources (e.g., road salts or wastewaters). Leopard frogs were historically one of the most widespread amphibian species but have declined in distribution in the Great Plains during the last 50 years (Corn and Fogleman, 1984; Kendell, 2004; Rorabaugh, 2005; Seburn and Seburn, 1998; Werner, 2003). Contaminants have been suggested as a contributing factor to declines of leopard frogs in the Canadian Great Plains (Wilson et al., 2008). Tiger salamanders and chorus frogs are generally common throughout their range, but their occurrence and abundance can be influenced by wastewater contamination in Montana and North Dakota within the PPR (Hossack et al., 2018).

To stock experiments, full or partial egg masses (chorus and leopard frogs) or early developmental stage larvae (tiger salamanders) were collected from 2 uncontaminated wetlands per location (< 32 mg Cl-/L, the lower limit of detection; Table 1). We collected tiger salamanders as larvae because we were unable to locate their eggs. We standardized collection of salamanders to a developmental stage of 12 because this was the earliest and most common stage at the time of collection (following the staging table of Watson and Russell, 2000). We measured Cl- concentrations of source waters where species were collected using Hach QuanTab Cl- titration test strips (ranges: 32–600 ["low"] and 300–6,000 ["high"] mg Cl-/L; Hach Co., Loveland, Colorado, USA). Test strips have a reported accuracy of ± 10%, but 30 samples tested with both titration strips and in the laboratory had a strong relationship with each other in a previous study ($R^2 = 1.00$; Hossack et al., 2018).

We conducted experiments over two years (2018 and 2019) and in different places in each year (US Fish and Wildlife Service Crosby Wetland Management District in North Dakota or University of Montana Fort Missoula Research Station in Montana, USA; Table 2), but laboratory and rearing conditions were the same (21 °C with a 14:10 h, light: dark cycle). Egg masses and larvae were placed in water from collection sites and kept cool with ice packs during transport to the laboratory. Tiger salamander larvae were held in individual 1-L containers and acclimated to laboratory conditions prior to initiating experiments. Eggs of frogs were reared in 15-L plastic tubs filled with 10 L of tap water treated with Tetra AquaSafe Plus to remove chloramines (following manufacturer instructions) and acclimated to room temperature. Chloride concentration of tap water was < 32 mg Cl-/L (the lower limit of detection). After hatching, frog larvae were fed Tetramin fish food *ad libitum*. Tiger salamander larvae were fed Black Worms (*Lumbriculus variegatus*) *ad libitum* prior to initiating experiments.

2.2. Acute toxicity experiments

We estimated acute toxicity across a wide range of environmentally relevant concentrations of Cl- for NaCl and wastewaters (generally 0–8,000 mg Cl-/L; Table 2) using standardized LC_{50} (96-h) experiments (herein, LC_{50} tests). To make clear comparisons between the two types of salinity, we used equivalent Cl- concentrations in wastewater and NaCl treatments. We conducted experiments in two years because of complete separation in the survival data for some species (all larvae were either alive or dead per concentration) that occurred in the first year, which caused problems fitting statistical models. Therefore, to fill in the gaps in exposure that occurred in year one, we conducted LC_{50} tests at smaller-

Table 1
Collection locations in Montana (MT) and North Dakota (ND), USA, for eggs (leopard and chorus frogs) and larvae (tiger salamanders). For 'Site,' WPA = Waterfowl Production Area and NWR = National Wildlife Refuge. Latitude and longitude coordinates are WGS84.

Species	Year	Site	State	Latitude	Longitude
Tiger salamander	2018	Rabenberg WPA	MT	48.84751	−104.13152
	2018	Norman WPA	ND	48.70719	−102.92881
Leopard frog	2018	Lostwood NWR	ND	48.67146	−102.48624
	2019	Pary WPA	MT	48.59028	−104.08496
Chorus frog	2018	Near Moran	WY	43.83300	−110.35500
	2019	Near Moran	WY	43.83300	−110.35500

Table 2

Locations and number of experimental units ('No. units') per type of treatment ('Type'; Wastewater or NaCl), nominal concentration (mg Cl-/L), and year for larval tiger salamanders, chorus frogs, and leopard frogs. 'Total units' describes the total number of experimental units in that subset. For 'Location,' CWMD = US Fish and Wildlife Service Crosby Wetland Management District and FMRS = University of Montana Fort Missoula Research Station. Ranges of nominal concentrations indicate a sequence of concentrations increasing at intervals of 1,000 mg Cl-/L.

Species	Year	Location	State	No. units	Total units	Type	Nominal concentration
Tiger salamander	2018	CWMD	ND	3	21	NaCl	0 and 2000–7000
Leopard frog	2018	CWMD	ND	10	90	Wastewater	0, 1000–8000
	2018	CWMD	ND	10	90	NaCl	0, 1000–8000
	2019	FMFRS	MT	10	30	Wastewater	0, 4200, 4600
	2019	FMFRS	MT	10	30	NaCl	0, 4200, 4600
Chorus frog	2018	CWMD	ND	4	36	Wastewater	0, 1000–8000
	2018	CWMD	ND	4	36	NaCl	0, 1000–8000
	2019	FMFRS	MT	4	16	Wastewater	0, 3500, 4500, 5500
	2019	FMFRS	MT	4	16	NaCl	0, 3500, 4500, 5500

concentration increments testing fewer concentrations in year two (Table 2). We collected salamander larvae and exposed them to a wide range of wastewater concentrations in 2019 but removed the data from our study because of high mortality in controls (US Environmental Protection Agency, 2002).

To make treatment solutions, we first treated room temperature tap water with Tetra AquaSafe Plus to remove chloramines (herein, lab water). For NaCl treatments, we created a stock solution of 20 g Cl-/L by mixing 33 g NaCl (ACS certified > 99.0% purity) per liter of lab water. Aliquots of the stock solution were diluted with lab water to match desired exposure concentrations. For wastewater treatments, we collected and mixed water from two highly contaminated sites within Rabenberg Waterfowl Production Area near Westby, Montana (each ~12,000 mg Cl-/L). We diluted aliquots of the wastewater mixture with lab water to meet desired exposure concentrations. We measured Cl- concentrations before and after the experiment using Hach QuanTab Cl- test strips. Experimental units were individual larvae in a ~300 ml plastic cup with 200 ml of treatment solution. We marked each cup at 200 ml and added lab water to maintain the volume in each cup throughout the experiment and avoid changes in Cl- concentration due to evaporation. We checked volumes regularly, but adding fresh lab water to individual cups was necessary < 5 times.

Measured concentrations for all treatments were close to targeted concentrations and did not differ between the beginning and end of experiments. Control treatments were always below detection limits for Cl- (< 32 mg Cl-/L). Mean concentrations of treatments for NaCl and wastewater were within 2.6% of nominal concentrations (95% confidence interval [CI] = 2.0–3.2%). Mean difference in Cl- concentrations between initiation and after experiments was -6 mg Cl-/L (95% CI = -29–17 mg/L).

We initiated experiments with tiger salamanders 48 h after capture (at a developmental stage of 12; Watson and Russell, 2000) to first acclimate them to laboratory conditions. We initiated experiments with frogs when larvae reached Gosner stage 25 (Gosner, 1960), which is when independent feeding begins. We selected these stages because we were unable to collect tiger salamander eggs and had to wait until salamanders hatched and larvae were available to capture. Although staging tables of larval salamanders and frogs have different scales, all larvae were at generally comparable stages and were free swimming and feeding. We also selected these stages because they match previous LC_{50} studies with NaCl and amphibians (e.g., Collins and Russell, 2009; Sanzo and Hecnar, 2006).

Larvae were randomly selected and assigned to each treatment combination (Table 2) and exposed to treatments for 96 h. Following standard acute toxicity protocols, we did not feed larvae during the experiment (ASTM Standard E47, 2008). We checked mortality at 6 h intervals for the first 24 h, and then every 24 h thereafter until terminating the experiment at 96 h. We considered larvae dead if they were unable to right themselves or were unresponsive to repeated prodding and removed them from the experiment. We ensured morality of larvae removed from the experiment and euthanized all live larvae at the end of the experiment (at 96 h) using MS-222 (tricaine methanesulfonate). We measured snout-vent length (herein, SVL; mm) and mass (g) of larvae that survived the 96-h exposures immediately following euthanization (n = 21, 115, and 55 for tiger salamanders, leopard frogs, and chorus frogs, respectively).

2.3. Statistical analyses

We used Bayesian generalized linear models ('bayesglm') in the package 'arm' (Gelman et al., 2018) to investigate the influences of wastewater, NaCl, or both on survival. We used Bayesian binomial logistic regression with a weakly informative Cauchy prior distribution because it enables convergence when partial or complete separation occurs (Gelman et al., 2008). Each individual was entered as alive or dead (1 or 0, respectively) with their respective covariates (concentration, treatment, SVL, mass, and location of experiment). For leopard and chorus frogs, we investigated the influence of concentration and treatment (wastewater or NaCl) separately per species and included a factor for location. We estimated concentrations at which 10, 50, and 90% mortality occurred (LC_{10}, LC_{50}, and LC_{90}, respectively) separately per species and treatment (NaCl or wastewaters) using the 'effects' package (Fox, 2003). We used linear regression to estimate the influence of concentration and treatment on size of larvae (SVL and mass) that survived 96-h exposures. For both the survival and size analyses for both frogs, we compared additive (concentration + treatment) and interactive (concentration × treatment) models with likelihood ratio tests ('lrtest' in the package 'lmtest'; Hothorn et al., 2019). We conducted all analyses in program R v3.6.3 (R Core Team, 2019).

3. Results

3.1. Toxicity of NaCl and energy-related wastewaters

Probability of survival of all species decreased with increased salinity ($p < 0.026$; Fig. 1A–C and Table 3) and survival was 100% in all control groups. Most mortalities in treatments occurred in the first 24 h of exposure. Additive models fit the survival data better than interactive models ($p > 0.609$; Table 4), which indicated that differences between NaCl and wastewater treatments were consistent across our range of concentrations. Probability of survival did not differ between NaCl and wastewaters for leopard frogs ($p = 1.00$; Fig. 1A&B and Table 3), but differed for chorus frogs ($p = 0.004$; Fig. 1C). For wastewaters, chorus frogs had a lower mean LC_{10} and LC_{50} value than leopard frogs, but leopard frogs had a lower mean LC_{90} (Table 5). For NaCl, leopard frogs had the lowest mean LC_{10}, LC_{50}, and LC_{90} values followed by chorus frogs and then tiger salamanders.

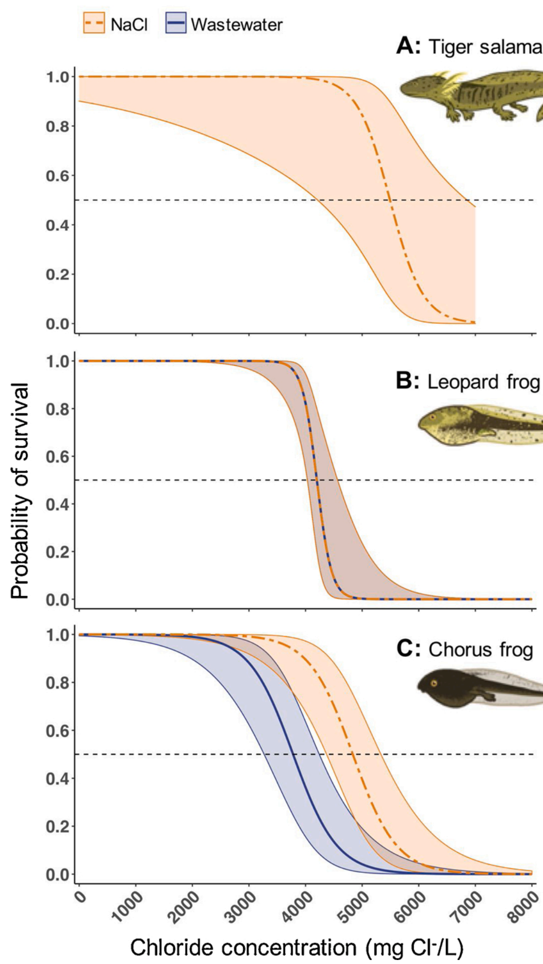

Fig. 1. Mean probability of survival (+/– 95% confidence intervals) after 96-h exposure to wastewaters (blue solid lines), sodium chloride (NaCl; orange dashed lines), or both for larval (A) tiger salamanders, (B) leopard frogs, and (C) chorus frogs. The dashed horizontal line indicates where 50% probability of survival (LC_{50}) occurs for each species. Probability of survival only differed between NaCl and wastewaters for chorus frogs.

3.2. *Sublethal effects of NaCl and energy-related wastewaters*

Increased salinity inhibited growth (SVL and mass) of leopard and chorus frogs, but not tiger salamanders (Figs. 2 and 3A–C and Table 3). Effects of increased salt concentration on SVL and mass did not differ between NaCl and wastewater treatments for chorus frogs ($p > 0.390$; Table 3). Mean mass and SVL of chorus frogs in controls was 1.58 times heavier and 1.21 times longer than those in 5,000 mg Cl-/L, respectively. However, for leopard frogs, exposure to NaCl decreased SVL and mass more than exposure to wastewaters ($p < 0.012$; Table 3). Mean mass of leopard frogs in controls was 1.79 and 1.31 times heavier than those in 4,200 mg Cl-/L NaCl or wastewater treatments, respectively. Mean SVL of leopard frogs in controls was 1.19 and 1.04 times longer than those in 4,200 mg Cl-/L NaCl or wastewater treatments, respectively. We did not quantitatively measure behavior but observed that larvae in higher concentrations of wastewater and NaCl (≥ 5,000 mg Cl-/L) were more lethargic, less responsive to prodding, and often found lying on their side on the bottom of their cup. A few larvae also had bent tails and flushed skin.

4. Discussion

4.1. *Toxicity of NaCl and energy-related wastewaters*

We exposed amphibian larvae from the Prairie Pothole Region (PPR)

Table 4
Summary statistics for likelihood ratio tests comparing interactive (concentration × treatment) and additive (concentration + treatment) models investigating the influence of treatment (wastewater or NaCl) and concentration (mg Cl-/L) on survival, snout-vent length (SVL), and mass of larval chorus and leopard frogs. All model comparisons differed by 1 degree of freedom. Bold values indicate where the interactive model was used instead of the additive model.

Species	Trait	χ^2	*p*
Leopard frog	Survival	0.01	0.917
	Mass	6.59	**0.010**
	SVL	15.12	**< 0.001**
Chorus frog	Survival	0.26	0.609
	Mass	0.65	0.421
	SVL	0.22	0.638

Table 3
Summary statistics for Bayesian generalized linear models (for survival; logit scale β's and standard errors [SE] with associated *z*-values) and linear models (for mass and snout-vent length [SVL]; normal scale β's and with associated *t*-values) investigating the influence of treatment (wastewater or NaCl) and concentration (mg Cl-/L) on survival, mass, and SVL of larval tiger salamanders, chorus frogs, and leopard frogs.

Species	Trait	Variable	*β*	SE	*df*	*z* / *t*	*p*
Tiger salamander	Survival	Concentration	−0.0034	0.0015	20	−2.23	0.026
	Mass	Concentration	−1.18E-05	1.60E-05	13	−0.73	0.476
	SVL	Concentration	−0.0003	0.0003	13	−0.89	0.392
Leopard frog	Survival	Concentration	−0.0077	0.0027	236	−2.86	0.004
		Treatment	1.70E-15	0.7633	236	0.00	1.000
	Mass*	Concentration	−1.60E-05	4.89E-06	110	−3.28	0.001
		Treatment	0.0147	0.0169	110	0.87	0.388
		Concentration × Treatment	−1.66E-05	6.50E-06	110	−2.55	0.012
	SVL*	Concentration	−0.0002	0.0001	110	−2.57	0.012
		Treatment	0.6330	0.2030	110	3.12	0.002
		Concentration × Treatment	−0.0003	0.0001	110	−4.23	< 0.001
Chorus frog	Survival	Concentration	−0.0026	0.0006	100	−4.41	< 0.001
		Treatment	2.7148	0.9378	100	2.90	0.004
	Mass	Concentration	−1.47E-05	5.44E-06	51	−2.70	0.009
		Treatment	−0.0027	0.0186	51	−0.14	0.886
	SVL	Concentration	−0.0002	0.0001	51	−2.31	0.025
		Treatment	−0.2864	0.3302	51	−0.87	0.390

Table 5
Estimated mean (+/− 95% confidence intervals) 96-h lethal concentration values (mg Cl-/L) for 10% (LC_{10}), 50% (LC_{50}), and 90% (LC_{90}) mortality for larvae exposed to wastewater or NaCl.

Species	Treatment	LC_{10}	LC_{50}	LC_{90}
Tiger salamander	NaCl	4850 (16–5485)	5492 (4212–6853)	6132 (5499–11130)
Leopard frog	Wastewater	3854 (3197–4021)	4141 (3947–4352)	4427 (4257–5125)
	NaCl	3854 (3197–4020)	4141 (3947–4352)	4427 (4257–5125)
Chorus frog	Wastewater	2925 (1972–3407)	3779 (3267–4241)	4633 (4316–5458)
	NaCl	3980 (3140–4434)	4834 (4371–5332)	5689 (5216–6616)

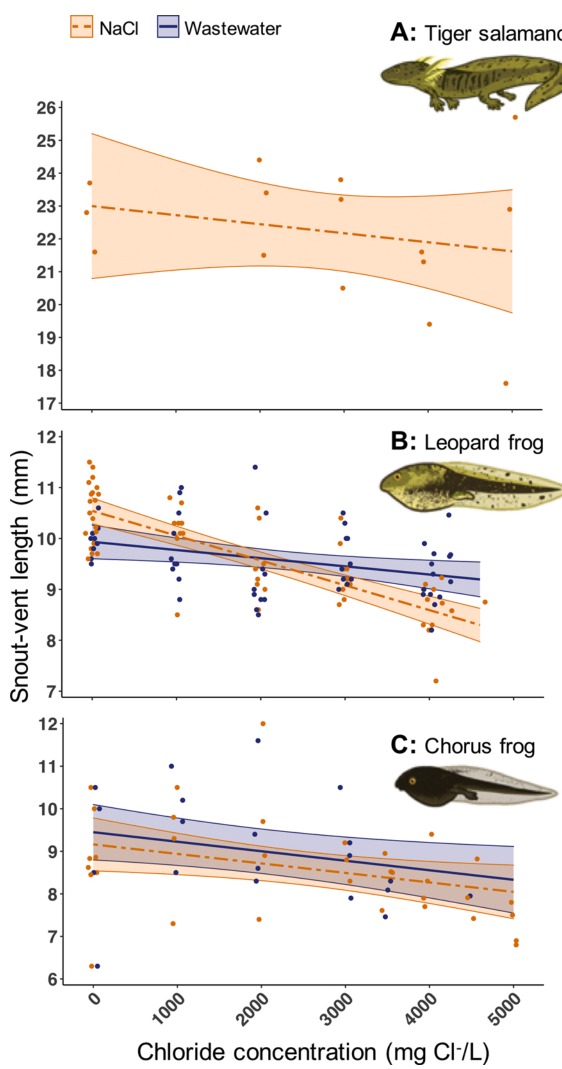

Fig. 2. Mean snout-vent length (SVL; +/− 95% confidence intervals) after 96-h exposure to wastewaters (blue solid line), sodium chloride (NaCl; orange dashed line), or both for larval (A) tiger salamanders, (B) leopard frogs, and (C) chorus frogs. The relationship between chloride concentration (mg Cl-/L) and SVL was statistically significant for leopard and chorus frogs and only differed between wastewaters and NaCl for leopard frogs.

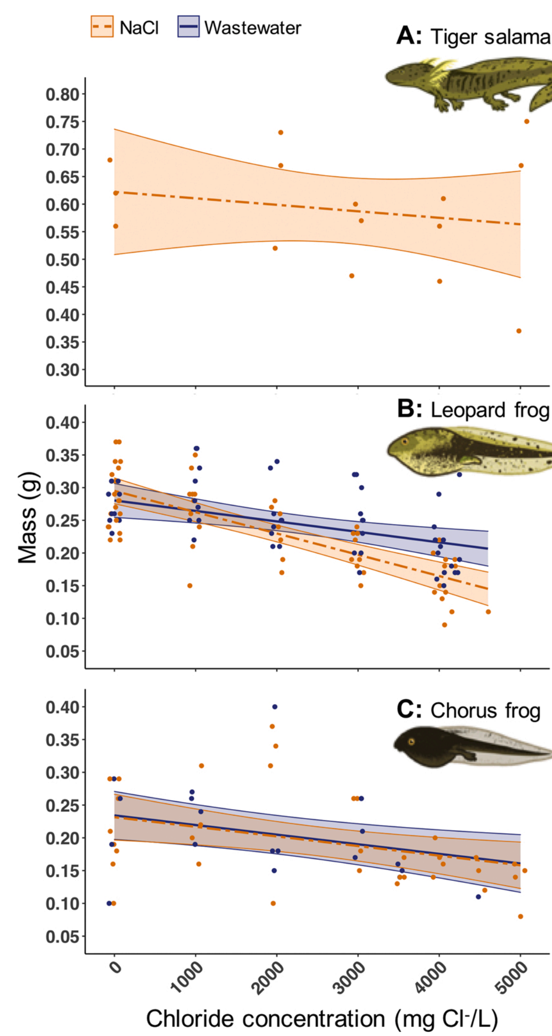

Fig. 3. Mean mass (+/− 95% confidence intervals) after 96-h exposure to wastewaters (blue solid line), sodium chloride (NaCl; orange dashed line), or both for larval (A) tiger salamanders, (B) leopard frogs, and (C) chorus frogs. The relationship between chloride concentration (mg Cl-/L) and mass was statistically significant for leopard and chorus frogs and only differed between wastewaters and NaCl for leopard frogs.

of North America to an environmentally relevant range of Cl- concentrations (0–8,000 mg Cl-/L) to determine the relative toxicity and sublethal effects of NaCl and wastewaters. Survival declined steeply between 4,000–5,000 mg Cl-/L of wastewater or NaCl for both frogs. For NaCl, tiger salamanders had the highest LC values and widest interval of Cl- concentrations between LC_{10} and LC_{90} values compared to both frogs. Chloride concentrations that were lethal to the species we studied can be far surpassed in areas contaminated by road salts (maximum 13,500 mg Cl-/L; Ohno, 1990) and wastewaters (maximum 31,660 mg Cl-/L in North Dakota; Gleason and Tangen, 2014). Therefore, contamination from road salts and wastewaters could make some aquatic ecosystems uninhabitable for the species we studied.

Mean LC_{50} values were similar for leopard and chorus frogs and only slightly higher for tiger salamanders, but all were higher than those documented for congeners of the same developmental stage (Gosner stage 25; Gosner, 1960). The median LC_{50} for Spring Peepers (*Pseudacris crucifer*) exposed to NaCl road salt was 2,830 mg Cl-/L (Collins and Russell, 2009), compared to a mean of 4,834 mg Cl-/L for chorus frogs (*P. maculata*) exposed to NaCl in our study. The median LC_{50} for Green Frogs (*Rana clamitans*) exposed to NaCl road salt was 3,100 mg Cl-/L

(Collins and Russell, 2009), compared to a mean of 4,141 mg Cl-/L for leopard frogs (*R. pipiens*) in our study. One of the only *Ambystoma spp.* with a known LC_{50} is the Spotted Salamander (*A. maculatum)*, which had a median LC_{50} value of 1,170 mg Cl-/L (Collins and Russell, 2009). In another study, larval tiger salamanders in Washington State could "tolerate" 10,290 mg Cl-/L, but these larvae were collected in high-salinity ponds between ~7,000–8,000 mg Cl-/L and had ostensibly acclimated prior to capture (Gasser and Miller, 1986). The LC_{50} value for tiger salamanders exposed to NaCl in our study was 5,492 mg Cl-/L, but these larvae were not previously acclimated to high-saline conditions.

The differences in tolerance for tiger salamanders and leopard frogs, compared to closely related species, could have resulted from local adaptions to naturally occurring salts or occurring in areas contaminated by wastewaters for many years. Some water bodies in the PPR (< 10%; Gleason and Tangen, 2014) are dominated by naturally occurring sulfate salts, and salinity can vary within and across years (SO_4^{2-}; e.g., magnesium, sodium, or calcium sulfate; Swanson, 1988). Chloride salts also naturally occur in North Dakota and Montana water bodies but have increased in the past 60 years because of wastewater contamination (Gleason and Tangen, 2014). Adaptations to saline conditions can evolve rapidly in amphibian populations (e.g., 40–70 years; Brady, 2012). Divergent and adaptive responses to salt exposure have been documented for several amphibian species. For example, coastal American Green Tree Frog (*Hyla cinerea*) and Rough-skinned Newt (*Taricha granulosa*) populations had higher tolerances or were less physiologically reactive to salinity than inland populations (Albecker and McCoy, 2017; Hopkins et al., 2016). However, the chorus frogs in our study were collected from an area without a history of secondary salinization and still had higher LC_{50} values than closely related Spring Peepers. Therefore, species-level comparisons for salt tolerance—even among congeners—may not be particularly informative. Further investigations are necessary to gain a more-complete understanding of inter- and intraspecific differences in salinity tolerances.

Larvae of the three species we studied occur in wetlands with wastewater contamination, but this varies by species with the maximum Cl- concentration up to ~5,000 mg Cl-/L for tiger salamanders (Hossack et al., 2018). However, concentrations that influenced amphibian abundance in wetlands were lower than concentrations that influenced survival in our study (Hossack et al., 2018). The differences between field and laboratory results may be related to the use of different metrics (abundance compared to survival), timing and length of time of exposure, or behavioral effects of Cl- that could increase vulnerability to predation in natural settings (Collins and Russell, 2009; Denoël et al., 2010; Hall et al., 2017; Kearney et al., 2016; Sanzo and Hecnar, 2006).

Only chorus frog survival was more sensitive to wastewaters than NaCl in our study. A previous study of chorus frog survival also indicated that they were more sensitive to wastewaters from the Williston Basin than to Cl- alone (Hossack et al., 2017). In studies that compared road salts to NaCl alone, larval amphibians were equally sensitive to road salts and NaCl despite NaCl being the predominant component of road salt and wastewaters (Jones et al., 2015; Winkler and Forte, 2011). We are uncertain why only one species was more sensitive to wastewater than NaCl but the capacity to tolerate increased NaCl, other contaminants in wastewaters, or conductivity may be species-specific and depend on differences in phylogeny, behavior, size, and physiology (Egea-Serrano et al., 2012; Hopkins and Brodie, 2015). For ionic and osmotic imbalances, physiological responses include changes in hormone regulation, plasma osmolarity, water uptake, and ATPase activation (Hopkins et al., 2016; Shoemaker and Nagy, 1977; Uchiyama and Konno, 2006; Ultsch et al., 1999; Warburg, 1995; Wu et al., 2014). Wastewaters from our study area (Williston Basin) contain various mixtures of chromium, lead, selenium, strontium, and other toxic heavy metals (Beal et al., 1987; Farag and Harper, 2014) that can be taken up and accumulate in amphibian tissues, dependent upon feeding traits (Smalling et al., 2019). Independently, metals can negatively influence sodium regulation in fishes (Sola et al., 1995; Wilson and Taylor, 1993) and survival and development in amphibians (Freda, 1991; Lanctôt et al., 2016; Lefcort et al., 1998; Weir et al., 2016). Conductivity of wastewaters in the Williston Basin are also typically higher than those of aqueous NaCl (Gleason and Tangen, 2014). Higher conductance may influence osmotic regulation of amphibians independent of NaCl concentration by interfering with ion or water exchange through the skin (Chambers, 2011; Ultsch et al., 1999).

4.2. Sublethal effects of NaCl and energy-related wastewaters

For frogs, growth of larvae that survived 96-hr experiments decreased with increasing concentrations of wastewater and NaCl. Smaller size of larvae can decrease subsequent fitness (Altwegg and Reyer, 2003; Cabrera-Guzmán et al., 2013; Semlitsch et al., 1988). Compensatory growth can sometimes offset potential costs for some amphibians (Capellan and Nicieza, 2007; Hector et al., 2012), but exposure to salt during late-larval stages can inhibit compensatory growth (Hall et al., 2017; Kearney et al., 2014). Effects of wastewater and NaCl differed only for leopard frogs. As for survival, this difference could be related to the additional contaminants in wastewaters or species-specific differences in tolerance to contaminants. It may also take longer than 96 h for differences in growth between NaCl and wastewaters to manifest for some species. The different responses could also be related to variation in our experiment because how species responded to wastewater or NaCl depended on the trait (i.e., we observed effects on survival or growth, but not both). Although effects of wastewater and NaCl were statistically different for leopard frogs, differences between the two treatments were small and may have indistinguishable effects *in situ*. Nevertheless, sublethal effects of wastewater seemed driven primarily by NaCl for chorus frogs because growth effects did not differ between wastewaters and NaCl.

Although anecdotal, increased salinity seemed to fatigue larvae and reduce their activity. Activity of several other amphibian species was also reduced when exposed to increased salinity (Collins and Russell, 2009; Denoël et al., 2010; Jones et al., 2017; Sanzo and Hecnar, 2006). Reductions in movement can reduce feeding, increase predation risk, and decrease survival (Collins and Russell, 2009; Dickman and Christy, 2002; Gomez-Mestre and Tejedo, 2003; Kearney et al., 2016). As discussed in Sanzo and Hecnar (2006), Cl- LC_{50} values for predators (e.g., invertebrates and fishes) are typically higher than those of larval-amphibian prey. Decreased ability of prey to respond to predators, combined with lower survival of prey relative to predators, could intensify the influences of salinity on amphibian populations.

5. Conclusions

Even though salinity is a ubiquitous anthropogenic stressor, we still have a limited understanding of the effects of freshwater salinization on vertebrate growth and survival (Brittingham et al., 2014; Hintz and Relyea, 2019; Souther et al., 2014). Our experiments with amphibian larvae illustrated that effects vary among species and Cl- concentrations and marginally between NaCl and wastewaters. Pairing experimental and field studies in the same study system is crucial to identify mechanisms and connect patterns of sensitivity, thereby enabling a more complete understanding of effects of stressors. Because effects of wastewater were predominantly due to NaCl, previous research on the effects of NaCl and road salts may be useful to guide conservation and remediation efforts in areas affected by energy development and other sources of secondary salinization. However, generalizations comparing effects of wastewater to NaCl on amphibians will require further investigations because this is only the second study to investigate amphibian tolerances for wastewaters. Despite the need for further investigations, our study provides benchmarks for acute toxicity of wastewater and NaCl contamination for these species. The persistence and negative influence of salinity in freshwater ecosystems demonstrates the need to restore affected landscapes and develop alternative solutions to reduce the salinization of freshwater ecosystems (Schuler et al., 2018).

CRediT authorship contribution statement

Brian J. Tornabene: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Funding acquisition. **Creagh W. Breuner:** Methodology, Writing - review & editing, Supervision, Funding acquisition. **Blake R. Hossack:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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