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Short Communication

Influence of Ion Deficiency in Freshwater Short-Term Chronic and Acute Toxicity Tests with *Ceriodaphnia dubia, Daphnia magna* and *Pimephales promelas*

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Abstract

While several studies have been focused on the toxicity of elevated concentrations of one or more of the major freshwater ions (e.g., Ca^{2+}, Na^{+}, Cl^{-}), fewer data are available on the adverse impacts of ion deficiency. Short-term chronic (STC) toxicity tests, with *Ceriodaphnia dubia*, *Daphnia magna*, and the fathead minnow, *Pimephales promelas*, were conducted using laboratory-prepared test waters representing a range of water quality conditions. In addition, acute studies of an ion-deficient (reverse osmosis) effluent were conducted with *C. dubia* and *P. promelas*. *Ceriodaphnia dubia* was the most tolerant of the three species in STC tests, with 50% survival in deionized water, while there was 100% mortality of the other two species. Reproduction of *C. dubia* was unaffected in any of the other reconstituted waters, even at a hardness of only 10 mg/L as $CaCO_3$. In treatments having surviving organisms, growth (biomass) of *D. magna* was significantly reduced in the softest water, while growth of *P. promelas* was significantly reduced in the two softest waters. In the acute studies, only *P. promelas* survival was significantly reduced in RO effluent. A modest increase in the hardness and alkalinity of the RO effluent eliminated the ion deficient effect, resulting in 100% survival. Based on these data, *D. magna* and *P. promelas* have similar sensitivities to ion deficiency. These studies demonstrate the importance of evaluating ion deficiency as well as excess when assessing wastewaters and receiving waters.

ABBREVIATIONS

WET: Whole Effluent Toxicity; RO: Reverse Osmosis; STC: Short-Term Chronic; TDS: Total Dissolved Solids; MQ: Milli-Q® Water; VSW: USEPA Very Soft Water; SW: USEPA Soft Water; MHW: USEPA Moderately Hard Water; HW: USEPA Hard Water; VHW: USEPA Very Hard Water

INTRODUCTION

The chemical characteristics of a body of water depend on the underlying watershed lithology, inputs of exogenous inorganic and organic materials and internal biochemical interactions [1]. Regardless of the characteristics unique to any given water body, most freshwater systems are dominated by seven ions: Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Cl^{-}, SO_4^{2-}, and HCO_3^{-}. Although these major ions play essential physiological roles in aquatic organisms [2,3], when present in excess concentrations they can cause significant acute and chronic toxicity to freshwater species This toxicity may be observed in organisms commonly used in Whole Effluent Toxicity (WET) tests, which are one of the required analyses in many discharge permits throughout the United States and other countries.

While the toxicity of elevated major ions to several species, and in particular those used in WET tests, has been well-studied [3,4], effects of deficient ion concentrations are less well known. Marine species are more likely to be affected by solutions with low osmotic strength and/or deficient concentrations of certain ions. This is especially true of stenohaline organisms that can tolerate only a limited range of salinities. Pillard et al. [5], evaluated the acute toxicity of major ions in seawater to mysid shrimp (*Americamysis bahia*), sheepshead minnow (*Cyprinodon variegatus*) and inland silverside minnow (*Menidia beryllina*). They found that while several ions were toxic when present in elevated concentrations, not all caused toxicity when present in very low concentrations. The most distinct increase in mortality to all three species as a result of ion deficiency occurred with Ca^{2+} and K^{+}. This information on the effects of deficient and excess ions to marine organisms was used to develop the Marine Salinity Toxicity Relationship (MSTR) program to aid researchers and compliance managers when facing toxicity in effluents released to the marine environment [6]. While similar quantitative information on the adverse effects of ion deficiencies in freshwater is generally lacking, it is no less important. Some effluents may have low ionic strength that can affect test organisms in WET tests, possibly leading to a false positive indication of toxicity. Reverse osmosis (RO) is an effective treatment for removing a variety of organic and inorganic molecules from wastewater, often leaving the resulting effluent very low in essential ions. Natural geochemistry in certain regions can also lead to very low mineral levels in surface and ground water; an example is the Piedmont region of the southeastern United States [7]. Effluents discharged in areas of soft (source) water may also be low in hardness, creating a potential for mis-interpretation of WET test results.

This study was conducted to assess the effects of low-TDS waters to freshwater species that are commonly used in WET tests: *Ceriodaphnia dubia*, *Daphnia magna* (Cladocera; Daphnidae), and *Pimephales promelas* (fathead minnow; Cyprinidae). Short-term chronic tests were conducted with each of these species using laboratory waters adjusted to various levels of ionic strength. In addition, acute studies were conducted with *C. dubia* and *P. promelas* using oil field effluent that had been treated with an RO system.

MATERIALS AND METHODS

Assessing chronic effects of ion-deficiency in freshwater toxicity tests

Short-term chronic (STC) toxicity tests were conducted with *C. dubia*, *D. magna*, and *P. promelas*. All three species were cultured at the TRE Environmental Laboratory. *Ceriodaphnia dubia* and *P. promelas* were cultured in USEPA Moderately Hard Water (MHW) and *D. magna* was cultured in USEPA Hard Water (HW). Test procedures for *C. dubia* and *P. promelas* are promulgated methods for WET tests as described in USEPA [8]. Each of these tests was conducted for seven days. The *D. magna* STC test is a 4-d procedure developed by Lazorchak [9], which is not a promulgated WET method but is a useful test procedure for monitoring effluent quality in selected cases. Organism survival was assessed each day of the test. Biomass (dry weight per initial number of organism) was the sub lethal endpoint for *P. promelas* and *D. magna*; reproduction (number of neonates produced in three broods) was the sub lethal endpoint for *C. dubia.* Six laboratory-prepared waters were tested, representing a wide range of freshwater ion concentrations: ASTM Type-I water (Milli-Q® water [MQ]), USEPA Very Soft Water (VSW), USEPA Soft Water (SW), USEPA Moderately Hard Water (MHW), USEPA Hard Water (HW), and USEPA Very Hard Water (VHW) [8]. MHW, commonly used as control and dilution water in WET tests, was identified as the control treatment. The reconstituted USEPA waters were prepared by adding reagent grade salts (as listed in [8]) to MQ water having a resistivity of > 18 MΩ.

Acute toxicity of RO waste water: application of ion add-back

Acute toxicity tests were conducted on wastewater from an oil production facility in the state of Wyoming, USA that uses an RO system to treat its effluent. The effluent was very low in hardness and alkalinity, with a conductivity of approximately 150 µS/cm. Because preliminary tests indicated that the effluent might cause significant mortality to test organisms, a study was conducted to determine if amending the effluent to raise the hardness and alkalinity to match that of MHW would improve organism performance. Both the ambient effluent and (ion-) enhanced effluent were tested using *C. dubia* and *P. promelas*. The acute test with *C. dubia* was 48 h in duration; the *P. promelas* acute test was 96 h in duration. Each was conducted according to USEPA acute methods [10].

Water chemistry

Hardness and alkalinity of all test solutions were measured by titration using Standard Methods (SM) 2340 C and SM 2320 B, respectively [11]. Temperature of the test solutions was measured using a calibrated thermometer and pH was measured using Oakton PCSTestr Model 35. Dissolved oxygen was measured using YSI Model 51B and specific conductance was determined using a ThermoScientific Orion Model 125.

Data analysis

Proportional survival data were transformed using arc-sine square root prior to analysis. One-way analysis of variance (ANOVA) was used to determine statistical differences at a significance level (α) of 0.05. Data were evaluated using one-tailed tests to identify treatments that were significantly less than the MHW control. When assumptions of normality and homogeneity of variance (α=0.01) were not met, nonparametric ANOVA tests were used. ANOVA was followed by appropriate multiple comparison tests. Statistical analyses were performed using CETIS v 1.8.7 [12].

RESULTS AND DISCUSSION

Assessing chronic effects of ion-deficiency in freshwater toxicity tests

Each of the tested waters had a distinctive ionic strength, as illustrated by increasing hardness, alkalinity and conductivity (Table 1). The MQ water had extremely low ion concentrations, as would be expected from a water treatment system yielding high-purity water. Hardness and alkalinity of the reconstituted USEPA waters increased roughly 20x between the softest water (VSW) and the hardest water (VHW). The nominal concentrations of major ions are based on the weight of salts added to prepare the waters. None of the ion concentrations were high enough to cause toxicity in excess [3].

Survival and sub lethal response of each species was acceptable in the MHW (control) treatment (Table 2). Survival was near 100% for all species in all waters with > 42 mg/L hardness. All *P. promelas* and *D. magna* died in MQ water, although *D. magna* mortality occurred faster, with only two organisms alive at 24 h and all dead by 48 h; 52.5% of *P. promelas* remained alive at 24 h, and all were dead by 72 h. These data suggest that in relatively brief exposure conditions, *D. magna* may be more vulnerable than *P. promelas* to ion deficiency, and the latter might be able to recover if moved to ion-enhanced water. *Ceriodaphnia dubia* were much more tolerant of MQ water, with 100% survival through day 5, and 50% survival at test termination. In VSW (hardness = 10 mg/L), both *C. dubia* and *P. promelas* showed very good survival (100% and 97.5%, respectively); survival of *D. magna*, however, was only 30% in VSW. Reproduction in *C. dubia* was significantly reduced only in MQ water. Growth in *P.*

Table 1: Characterization and nominal ion concentrations of six waters used in short-term chronic studies.

Water	Initial pH	Hardness (mg /L as $CaCO_3$)	Alkalinity (mg /L as $CaCO_3$)	Specific Conduct. (µS/cm)	Nominal Conc. of Major Ions (mg/L)						
					Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	Cl^{-}	SO_4^{2-}	HCO_3^{-}
MQ	8.0	<2	1	1.3	<0.1	<0.1	<0.1	<0.01	<0.01	<0.1	1.22
VSW	7.6	10	12	40	1.98	1.71	5.10	0.09	0.07	11.5	14.6
SW	7.8	42	37	151	7.25	6.29	14.7	1.17	0.88	42.2	45.1
MHW	8.2	92	64	306	14.0	12.1	26.3	2.1	1.9	81.4	78.0
HW	8.0	162	110	533	28.0	24.3	52.4	5.42	4.09	163	134
VHW	8.0	284	224	945	49.4	42.9	107	9.81	7.41	288	273

Abbreviations: MQ: Milli-Q Water; VSW: USEPA Very Soft Water; SW: USEPA Soft Water; MHW: USEPA Moderately Hard Water; HW: USEPA Hard Water; VHW: USEPA Very Hard Water

promelas was significantly reduced in MQ, VSW and SW; growth of *D. magna* was significantly reduced in MQ and VSW (Figure 1). The sub lethal growth responses suggest *P. promelas* may be somewhat more sensitive than *D. magna* to ion deficiency during chronic exposures, although the more rapid mortality of the latter, as well as lower survival in VSW indicates the sensitivities of the two species are similar.

Acute toxicity of RO wastewater: application of ion add-back

Hardness of the ambient RO effluent was <2.0 mg $CaCO_3$/L and alkalinity was 8 mg $CaCO_3$/L, considerably lower than MHW (Table 3). Addition of laboratory salts raised the hardness and alkalinity of the effluent to be approximately equivalent to MHW (Table 3). The ambient RO effluent was toxic to *P. promelas*, with only 8 of 40 original fish (20%) surviving at 96 h, and an median lethal concentration (LC_{50}) of 83.84% effluent (Table 4). Since most acute limits in discharge permits do not allow a mixing zone, the allowable LC_{50} must be >100% effluent. The ambient RO effluent, therefore, failed the permit limit. In the ion-adjusted effluent, however, survival of *P. promelas* was 100% after 96 h; the ion-adjusted effluent passed the permit effluent limit. *Ceriodaphnia dubia* survival at 48 h was not significantly reduced in the ambient RO, indicating that it was less sensitive to ion deficiency than the fathead minnow, in this experimental exposure scenario.

Toxicity due to ion deficiency may be associated with a general intolerance of an extremely hypotonic environment, or the effects associated with the absence of specific essential ions. Calcium, for example, is one of the most important ions in biochemical reactions, serving in roles such as controlling the gating of Na^+ fluxes in the nerve membrane and regulation of cell membrane permeability by cross-linking adjacent phosphate groups between phospholipid molecules [3]. Mount et al. [13], identified atypical osmolarity as one of the mechanisms for toxicity to freshwater organisms when ion concentrations are in excess. Erickson et al. [14], reported similar results, noting a mechanism for non-specific ion toxicity when all ions contribute on an additive basis.

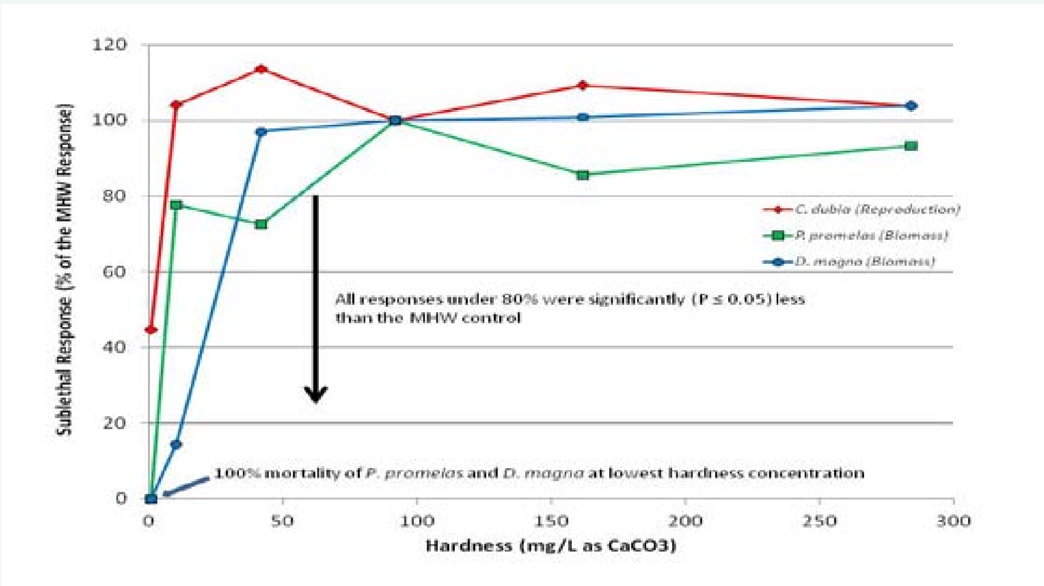

Figure 1 Sublethal response of *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimephales promelas* exposed to six waters representing increasing concentrations of major freshwater ions. Results are presented as a percent of the control response, the control being Moderately Hard Water (MHW, hardness = 92 mg/L).

Table 2: Survival and sublethal response of *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimephales promelas* in short-term chronic studies with six waters representing increasing concentrations of major freshwater ions, (represented by hardness).

Water Hardness (mg/L as $CaCO_3$)	*Ceriodaphnia dubia*		*Daphnia magna*		*Pimephales promelas*	
	Mean Survival	Mean # of Neonates (± S.E.)	Mean Survival (± S.E.)	Mean Dry Wt. (mg) (± S.E.)	Mean Survival (± S.E.)	Mean Dry Wt. (mg) (± S.E.)
<2	50	13.8 (± 1.98)	0	0	0	0
10	100	32.2 (± 1.81)	30 (± 10)	0.015 (± 0.006)	97.5 (± 2.5)	0.532 (± 0.023)
42	100	35.1 (± 1.97)	100	0.101 (± 0.002)	95 (± 2.9)	0.496 (± 0.029)
92	90	30.9 (± 4.34)	100	0.104 (± 0.001)	100	0.684 (± 0.021)
162	100	33.8 (± 1.24)	95 (± 5)	0.105 (± 0.004)	97.5 (± 2.5)	0.586 (± 0.050)
284	100	32.1 (± 1.08)	100	0.108 (± 0.002)	97.5 (± 2.5)	0.639 (± 0.037)

Note: Each replicate of the *C. dubia* short-term chronic test includes only one organism; therefore it is not possible to calculate a S.E. for survival

Table 3: Characteristics of control water and RO effluent, before and after amendment with laboratory salts.

Water	Initial pH	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Specific Conduct. (µS/cm)
Control Water (MHW)	8.4	88	57	306
RO Effluent	8.2	<2.0	8	130
RO Effluent + Ions	8.2	88	61	406

Abbreviations: RO: Reverse Osmosis; MHW: USEPA Moderately Hard Water

Table 4: Survival of *Ceriodaphnia dubia* and *Pimephales promelas* in acute studies with ambient RO effluent and RO effluent plus added ions.

Test Concentration (% Effluent)	Mean 48-h Survival (%) of *Ceriodaphnia dubia* (± S.E.)		Mean 96-h Survival (%) of *Pimephales promelas* (± S.E.)	
	Ambient RO	RO + Ions	Ambient RO	RO + Ions
0 (MHW)	100	100	100	100
12.5	95 (± 5)	100	100	100
25	100	100	100	100
50	100	100	100	100
62.5	100	100	100	100
100	95 (± 5)	100	20 (± 7.1)	100
LC_{50} (95% C.L.) (% Effluent)	>100	>100	83.84 (80.04 – 87.83)	>100

Abbreviations: RO: Reverse Osmosis; MHW: USEPA Moderately Hard Water; LC_{50}: Median lethal concentration

However, when concentrations of certain cations exceed given levels, other ion-specific mechanisms come into play [13,14]. The importance of hardness, and specifically the concentrations and ratios of Ca^{2+} and Mg^{2+}, has been demonstrated in single-ion and binary mixture studies [13,14]. Hardness in control waters or effluents does appear to affect organism performance at very high, and possibly very low, concentrations. In water with hardness at about 300 mg/L as $CaCO_3$, fathead minnow weight was significantly increased [15]. If a high-hardness receiving water is used as dilution water in a WET test with an effluent of much less hardness, significant differences in fish growth could arbitrarily result in toxicity when, in fact, the differences are simply an artifact of the influence of dilution water hardness [15].

CONCLUSION

In the current studies, the relative sensitivity to ion deficiency in STC tests is *P. promelas* ≈ *D. magna* > *C. dubia*. *Ceriodaphnia dubia*, which was cultured in MHW (with a hardness ranging from 90 to 100 mg/L), was notably more tolerant of ion deficiency in both the acute and STC studies, exhibiting strong reproduction in soft water with a hardness as low as 10 mg/L as $CaCO_3$.These results contrast with other studies which found that *C. dubia*, cultured at a hardness of 100 mg/L, had significantly reduced reproduction when tested in waters with a hardness of 45 mg/L [16]. Those significant sub lethal differences disappeared when *C. dubia* were cultured in soft water before being tested in soft water [16]. Variability in the *C. dubia* sub lethal response is common and could contribute to differences among studies. However, anecdotal data from the TRE Environmental Laboratory suggest that *C. dubia* are consistently tolerant of low-hardness effluents and receiving waters.

These studies confirm that ion deficiency can influence the outcome of toxicity tests using some of the most typical freshwater WET organisms. In soft water effluents and receiving waters, low ion concentrations could lead to significant effects and possibly a finding that toxicity is present. Understanding the impact and relative sensitivity of WET test organisms is important in separating effects due to background chemistry from effects due to the presence of a chemical stressor introduced by a particular manufacturing or treatment process. The current studies examined only ion deficiency as a whole, without consideration of ion ratios (ratios were generally maintained according to USEPA water recipes [8]). Additional studies that consider factors such as Ca:Mg ratios, Na^+ levels and test organism age will help to elucidate the importance of key essential ions in chronic and acute studies.

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