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Short communication

Implications of pH manipulation methods for metal toxicity: Not all acidic environments are created equal

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ABSTRACT

The toxicity of many metals is impacted by environmental pH, through both competition and complexation by hydroxide and carbonate ions. To establish safe environmental regulation it is important to properly define the relationship between pH and metal toxicity, a process that involves manipulating the pH of test water in the lab. The current study compares the effects of the three most common pH manipulation methods (carbon dioxide, acid–base addition, and chemical buffers) on acute Pb toxicity of a model fish species, *Pimephales promelas*. Acidification of test water revealed that the Pb and Pb^{2+} LC50 values were impacted by the pH manipulation method, with the following order of effects: HCl < CO_2 < MOPS. Conversely no differences in toxicity were observed when test pH was alkalinized using MOPS or NaOH. The different impacts of pH manipulation methods on Pb toxicity are likely due to different physiological stresses resulting from the respective methods; the physiological implications of each method are discussed. The results suggest that when studying the impacts of pH on metal toxicity it is important to properly replicate the ambient conditions of interest as artificial buffering using CO_2 environments or organic buffers significantly affects the physiology of the test organisms above and beyond what is expected from pH alone. Thus, using CO_2 and organic buffers overestimates the impact of acid pH on Pb toxicity.

Establishing safe environmental regulations for metals in aquatic ecosystems depends on correctly defining relationships between water chemistry and toxicity. The Biotic Ligand Model (BLM) is a commonly used approach that integrates the speciation, complexation, and competitive interactions of metals, water chemistry components and biotic ligands with toxicity endpoints to estimate effects in a wide range of aquatic habitats (Di Toro et al., 2001; Santore et al., 2001). A crucial step within this framework is to assess the impacts of isolated water chemistry components on toxicity. An important parameter for many metals is pH, as it shifts speciation to or away from toxic metal species; usually the free metal ion. In general a lower pH will increase free metal ions, while more alkaline pH will result in more carbonate complexes and fewer toxic free metal ions (Wood, 2012). Environmental pH can also impact toxicity through proton competition, where free protons compete with metals for binding sites on the biotic ligand or other complexing ligands such as dissolved organic carbon (DOC). This has been demonstrated for several metals including lead (Mager et al., 2011), copper (De Schamphelaere and Janssen, 2004; Deitmer, 2000; Schwartz and Vigneault, 2007), nickel (Deleebeeck et al., 2009; Pyle and Couture, 2012), zinc (Santore et al., 2002) and possibly cadmium (McGeer et al., 2012).

There are three different methods commonly used to manipulate pH during toxicity testing: (1) CO_2, (2) chemical buffers of specific pH, or (3) acid/base addition. All three methods successfully manipulate pH, but they also represent very different physiological stresses which have not previously been evaluated in a systematic comparison. Since toxicity is a combination of the physiological impacts of the toxicant and the environment, it is important to account for the distinct physiological stresses experienced by aquatic organisms exposed to the three pH manipulation methods. In this study we examine the impacts of pH manipulation methods on acute 96 h lead toxicity in the fathead minnow (*Pimephales promelas*). Lead (Pb) was chosen as a representative metal due to the well described effects of pH on acute toxicity in *P. promelas* (Esbaugh et al., 2011; Mager et al., 2011). In the present report, we compare Pb toxicity in previous studies performed in our lab using a MOPS buffer or HCl/NaOH addition (Grosell et al., 2006), with new data using CO_2 to control pH.

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Acute 96 h toxicity tests were performed on 8-day-old *P. promelas* using a previously described flow through set-up (Grosell et al., 2006; Mager et al., 2011). Animals were obtained commercially (Aquatic Biosystems, Fort Collins, Colorado) as 24 h-old-larvae and acclimated to test medium over a 7-day period. Animals were fed daily with *Artemia nauplii* (ad libitum) during the acclimation period, but were fasted during testing. Test medium consisted of dechlorinated Miami-Dade tap water diluted 2:1 with deionized water (22 °C). A commercially available DAQ-S pH/PCO_2 feedback system (Loligo Systems) was used to acidify test medium in both the primary and secondary mixing chambers using pure CO_2, as described previously (Esbaugh et al., 2012b; Heuer et al., 2012). The feedback pH probe was located in the no Pb control secondary mixing tank. Test pH was measured daily in one replicate of each concentration using a separate combination glass electrode coupled to a PHM220 pH meter (Radiometer). Water samples were taken daily from two replicates of each concentration to measure dissolved Pb. All samples were passed through a 0.45 μm Versapor membrane filter and acidified (1% HNO_3) prior to measurement, and analysis was performed using a graphite furnace atomic absorption spectrophotometer (AAS; Varian). Pb speciation calculations were performed using the acute Pb BLM (version 2.2.7; HydroQual, Inc.). Samples were taken for water chemistry analysis at 48 h and 96 h. Cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) were analyzed using flame AAS (Varian), while anions (Cl^- and SO_4^{2-}) were measured using ion chromatography (Dx-120; Dionex). Titratable alkalinity was determined using a double endpoint titration procedure (Hill, 1973). All statistical analyses were performed according to EPA guidelines for acute toxicity testing (USEPA, 2002) using the CETIS software package.

The water chemistry parameters for the CO_2 and concurrently run control toxicity test, as well as our previously performed MOPS (Grosell et al., 2006) and HCl/NaOH (Mager et al., 2011) tests are shown in Table 1. Despite our best efforts, there were some differences in water chemistry between test types. Most notable is the MOPS test series, which had approximately 75 μM less calcium than the acid–base or CO_2 treatment. The CO_2 treatment also had more DOC and sodium than the other two treatments. While sodium is not thought to affect Pb toxicity in fish over the range observed in this study, both calcium and DOC are crucial (Esbaugh et al., 2012a, 2011; Grosell et al., 2006; Mager et al., 2010, 2011). For this reason the impacts of pH manipulation method were deduced using the relative impacts on toxicity versus the pH method control group (pH 7.4 or 7.5). Note that water chemistry within pH method treatments varied little, with the exception of the ions added in conjunction with the buffers, acids and bases.

There were dramatic differences in the relative effects of the different pH manipulation methods used to acidify exposure waters (Table 2). The relative dissolved Pb LC50 was highest using the acid-base method with the acidified treatment LC50 dropping to 42% that of the control treatment. The relative Pb LC50 decreased to 26% of control values using the CO_2 method, and 15% using the MOPS method. These differences are even more pronounced when expressed as the Pb^{2+} LC50 (Table 2). Acidification using acid resulted in an increase in the relative Pb^{2+} LC50 when compared to neutral pH, demonstrating proton competition with Pb ions at low pH. However, neither the CO_2 nor MOPS methods showed similar findings as acidification resulted in either no change (CO_2), or a relative decreases in Pb^{2+} LC50 (MOPS). While Pb^{2+} toxicity did not appear to change as a consequence of pH when acidifying with CO_2, the absence of proton competition is representative of increased Pb^{2+} toxicity relative to the acid addition method, since the proton source is unlikely to impact proton–ligand binding dynamics.

The relative increase in Pb^{2+} toxicity at low pH observed for MOPS and CO_2 manipulation methods likely stems from an induced physiological stress (Fig. 1). With respect to MOPS, the

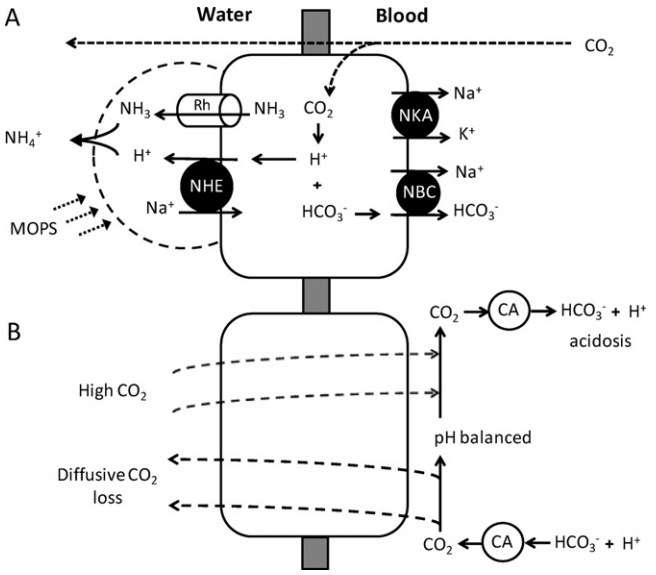

Fig. 1. A schematic of the physiological implications of pH manipulation methods on branchial ion exchange, acid-base balance and respiratory gas transport. (A) Electroneutral sodium uptake in freshwater is achieved by coupling RhCG1 mediated ammonia excretion with a co-localized Na^+/H^+ exchanger (NHE) on the apical membrane. Excreted ammonia buffers protons and creates an alkaline pH micro-domain, which eases the thermodynamic constraints of Na^+ transport allowing uptake from freshwater via NHE. Basolateral Na^+ transport occurs through both the Na^+/K^+-ATPase (NKA) and Na^+ HCO_3^- co-transporter (NBC). When in solution MOPS will prevent the alkaline micro-domain from forming by donating protons to maintain the acidic pH, thereby inhibiting Na^+ uptake as well as ammonia excretion. (B) During branchial capillary transit plasma HCO_3^- is transported into the red blood cell and hydrated to form CO_2; a reaction catalyzed by carbonic anhydrase (CA). CO_2 then diffuses across the branchial epithelium according to its concentration gradient. Hypercapnia exposure results in a reduced or reversed diffusive gradient that leads to blood CO_2 retention and a subsequent respiratory acidosis.

physiological stress can be attributed to disruption of the pH micro domain located at the apical membrane of freshwater fish ionocytes. These pH domains are critical in establishing the thermodynamic conditions for ion uptake from a freshwater environment. The most obvious example is Na^+ uptake through the Na^+/H^+ exchanger (NHE), which is a thermodynamically constrained process (Parks et al., 2008). Na^+ uptake via NHE relies heavily on ammonia efflux through an Rh channel to establish an alkaline pH domain (Fig. 1a) without which NHE cannot function (Kumai and Perry, 2011; Zimmer et al., 2010). Conversely, continued ammonia efflux relies on the presence of sufficient protons to convert NH_3 to NH_4^+ in the apical micro domain. Interestingly, exposure to HEPES buffers has been shown to interfere with Na^+ uptake in larval zebrafish reared at pH 4, presumably by increasing the buffer capacity of the micro domain (Kumai and Perry, 2011). In addition, MOPS has been shown to inhibit branchial sodium uptake and ammonia excretion (Shih et al., 2008, 2012).

The effects of elevated environmental CO_2 (hypercapnia) on fish are also well documented. Hypercapnia is a known stressor that inhibits CO_2 excretion by altering CO_2 diffusion gradients resulting in a respiratory acidosis that stimulates acid–base compensation mechanisms to protect intracellular and plasma pH (Fig. 1b). Because fish PCO_2 is much lower than that of air-breathing vertebrates due to their oxygen based ventilatory drive and the high solubility of CO_2 in water, even small changes can impact CO_2 transport. In fact, recent evidence suggests that PCO_2 levels as low as 1–2 matm can cause acid–base and osmoregulatory disturbances (Esbaugh et al., 2012b; Heuer et al., 2012). For perspective, the current CO_2 treatment is estimated to have a PCO_2 of at least 17 matm, as calculated using CO2SYS (freeware; Brookhaven National Laboratory). These CO_2 levels will establish an almost immediate blood

Table 1
Water chemistry parameters for 96-h acute lead toxicity tests for *Pimephales promelas*. For CO_2 data, ion concentrations are the mean of initial and final samples while pH is the mean (±SEM) of daily monitoring in all exposure concentrations.

Treatment		Na^+ (μM)	Ca^{2+} (μM)	Mg^{2+} (μM)	K^+ (μM)	Cl^- (μM)	SO_4^{2-} (μM)	DIC (μM)	DOC (μM)
Method	pH								
MOPS[a]	7.4	352	143	43	27	404	47	283	100
	6.7	358	157	34	222	541	30	209	100
	8.1	368	160	33	1672	305	30	303	100
Acid–base[b]	7.5	579	234	63	10	415	43	514	104
	6.3	561	226	61	10	903	41	24	118
	8.3	1050	186	60	10	577	48	667	123
CO_2	7.4 ± 0.02	938	236	66	10	512	39	518	172
	6.3 ± 0.02	934	216	59	12	433	40	533	166

[a] Grosell et al. (2006).
[b] Mager et al. (2011).

Table 2
The effects of three methods of pH manipulation on acute 96-h lead toxicity in the larval fathead minnow, *Pimephales promelas*.

	pH	Dissolved Pb LC50 (μg l^{-1})	Relative change (%)	Pb^{2+} LC50 (μg l^{-1})	Relative change (%)
MOPS[a]	7.4	52 (0.04–131)	–	0.23[c]	–
	6.7	8 (7.5–8.3)	15	0.09[c]	40
	8.1	15 (3–52)	29	0.01[c]	4
Acid–base[b]	7.5	624 (444–891)	–	32	–
	6.3	265 (149–416)	42	43	133
	8.3	340 (282–410)	54	1.1	4
CO_2	7.4	241 (193–296)	–	2.03	–
	6.3	62 (37–88)	26	2.02	99

[a] Grosell et al. (2006).
[b] Mager et al. (2011). Speciation re-calculated.
[c] Calculated from Pb LC50 and water chemistry values reported in Grosell et al. (2006).

and tissue acidosis, as previously shown for a number of fish species (e.g. Baker et al., 2009; Brauner et al., 2004; Crocker and Cech, 1998; Gilmour and Perry, 1994; Perry et al., 2010; Tzaneva et al., 2011), and initiate a number of energy consuming compensation pathways. Of course environmental acidosis will also result in blood acid–base disturbances due to diffusive ion movement; however, this is not of a respiratory origin and can be at least partially compensated for by respiratory mechanisms.

The impacts of treatment type on Pb toxicity were less when increasing, rather than decreasing environmental pH. Although the MOPS method decreased the dissolved Pb LC50 to a greater extent than direct base addition (Table 2), this apparent effect disappeared when expressing LC50s as ionic Pb^{2+} activities. While it is possible that pH micro domains may play a role in chloride uptake through anion exchangers, it appears that alkaline MOPS does not have the physiological impact of acidic MOPS in *P. promelas*. It is interesting that Pb toxicity increased with increasing pH in both treatments, with the ionic Pb^{2+} LC50 dropping to 4% that of neutral pH for both methods. So while alkaline pH may protect against Pb toxicity through complexation processes, the relative impact of ionic Pb^{2+} also increases. This may suggest a secondary toxic Pb species; however, the available evidence does not support this assumption (Esbaugh et al., 2011). Instead it appears that alkaline pH may affect the ability of calcium to compete with Pb for biotic ligand binding sites. Previous work has described a strong relationship between Pb^{2+} LC50 and calcium concentration for neutral and acidic waters (Esbaugh et al., 2011). The profile of this relationship changes in alkaline waters, possibly due to calcium complexation processes.

In conclusion, this study demonstrated that the pH manipulation method chosen can impact Pb toxicity by inducing physiological stress. While we have demonstrated impacts on Pb toxicity, these results are likely similar for many metals because the impact is irrespective of the metal, but is instead dependent on the physiology of the organism. It is clear that when selecting a pH manipulation method one must consider the environment it is attempting to replicate. For example, using CO_2 would only be valid for high CO_2 environments. We recommend that direct acid–base addition be used for most circumstances, as it is rare for regionally high PCO_2 to result in dramatic pH drops. We recommend against the use of buffers as they are detrimental to ionoregulatory processes at the apical gill membrane, and are not representative of the natural environment. While this has only been shown definitively for acidic buffers, it remains possible that alkaline buffers may impact some organisms. Improper selection of a pH manipulation method could result in overestimates in toxicity under acidic/alkaline pH conditions, and result in poor predictive success when modeling toxicity as a function of environmental pH.

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