Holland A et al., Annals of Environmental Science / 2013, Vol 7, 49-58

HUMIC SUBSTANCES INCREASE THE SURVIVORSHIP RATES OF FRESHWATER SHRIMP EXPOSED TO ACIDIFIED WATERS OF VARYING HARDNESS

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Received February 26, 2013; in final form May 22, 2013, accepted June 7, 2013.

ABSTRACT

Humic substances (HS) in naturally acidic waterways have been suggested to provide protection to aquatic organisms exposed to low pH conditions. Despite this, little is known about the ability of HS to increase survivorship of freshwater organisms as pH decreases in waters of varying hardness. This study explored the ability of HS in the form of Aldrich humic acid (AHA) to increase survivorship of the freshwater shrimp *(Caridina sp.* D) at low pH in artificial soft (representative of naturally acidic environments) and hard waters (artificial and natural Dee River water). Freshwater shrimp were exposed to pH treatments ranging from pH 7 to pH 3.5, with and without 10 or 20 mg/L HS treatments. In low pH water, shrimp mortality was higher in artificial hard water (LC_{50} at pH 4.95) and natural hard water (LC_{50} at pH 4.74), compared with soft water (LC_{50} at pH 4.27). HS substantially decreased the threshold at which pH caused 50% mortality to the freshwater shrimp, with that threshold shifting from 4.95 to 4.47 in artificial hard water, from 4.74 to 4.50 in natural hard water and from 4.27 to 4.18 in soft water. The results of this study are valuable in contributing to an improved understanding of the influence HS and water hardness has on the toxicity of low pH to freshwater biota and may have important implications for the management of acidified waterways.

Keywords: Dissolved organic carbon, toxicity, acid stress, hard water, soft water, naturally acidic

1. INTRODUCTION

Decreases in the pH of freshwater systems due to anthropogenic activities is a global problem. Increases in H^+ ion concentration cause changes to the regulation of ions in aquatic organisms and can lead to mortality of sensitive species, decreased diversity and poor ecosystem functioning. In contrast, waterways that are naturally acidic are able to maintain good diversity levels, with some examples even comparing well with diversity recorded from circumneutral waters [1-3]. Naturally acidic waterways are characterised by soft water with low buffering capacity, low pH and high organic carbon content. Unlike anthropogenically-acidified waterways, where the acidity is generally derived by inorganic means, the acidity of naturally acidic systems is caused by high levels of organic humic substances (HS) leached from the surrounding vegetation [3].

The presence of high amounts of HS in naturally acidic waterways may influence the impact pH has on aquatic organisms. For example, HS may buffer organisms against the detrimental effects of low pH in these systems [1,4]. Species diversity in waterways with pH < 5.7 has been shown in Europe to increase in water of intermediate or high humic content compared with water of similar pH value containing small amounts of HS [5-8] and in New Zealand [1,9,10]. In the Rio Negro, a naturally acidic river in Brazil, the presence of a high amount of dissolved organic carbon especially HS, is believed to prevent the ionoregulatory disturbance induced by decreases in pH. Possibly, this may occur by stimulating the uptake of essential ions, such as Na^+ and Ca^{2+}, thereby enabling aquatic biota to survive in these systems [11-13]. Improvements in growth and swimming behaviour of the larval striped marsh frogs (*Limnodynastes peronii*) have been recorded in the presence of HS in naturally acidic wallum water [14]. The survival rate of the freshwater amphipod *Gammarus pulex* to low pH in soft water was also increased with the addition of humus [15]. More recently, Serrano et al. [16] and Laudon et al. [17] have

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shown that the pH threshold of brown trout in naturally acidic waters rich in DOC is lower than that previously established for low DOC systems. These studies suggest a positive effect of naturally acidic water containing high amounts of HS against the detrimental effects of low pH.

Differences in water hardness have also been shown to play a role in determining the toxicity of a range of environmental stressors such as heavy metals. In regard to decreases in pH, elevated calcium levels that are characteristic of hard waters have been reported to protect organisms against increases in ion loss brought about by low pH [11,18]. Elevated calcium has also been suggested to lower the permeability of fish gills, thereby decreasing the rate of ions lost across their membranes [19]. Despite this, little is known about whether HS will increase survivorship of aquatic organisms in both soft and hard waters. This paper aims to investigate the ability of HS to increase survivorship of the freshwater shrimp (*Caridina sp. D sensu*; Page et al. [20]) to low pH in both soft water, which is characteristic of naturally acidic waters, as well as hard water environments, which are likely to be influenced by inorganic acidification. In doing so, the study can provide further insights into why naturally acidic freshwaters harbour more diverse communities than freshwaters acidified by anthropogenic means.

2. MATERIALS AND METHODS

Shrimp were collected from a site along the Dee River, Central Queensland, using a 250 µm dip net. The Dee River is characterised by a circumneutral pH in the range 7 to 8. At the time of collection the pH was approximately 7.3. On arrival in the laboratory, shrimp were placed in acclimation tanks containing either hard, soft or Dee River water (depending on the trial they were to be used in), within a controlled climate room at 25ºC and 16:8-h light:dark photoperiod for at least 48 hr before commencing experimental trials. The size of shrimp ranged from 1.6 - 2.1 cm in length from the tip of the cephalothorax to the end of the tailfin.

Test waters consisted of artificial soft water, artificial hard water or raw Dee River water. The artificial soft water was prepared using the recipe of Barth and Wilson [14] to mimic the ionic composition of naturally acidic (wallum) waters. Artificial hard water was prepared from the 'Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians [21]. Dee River water was collected at the same time as the test organisms and stored in 10 litre plastic containers in the dark both before and after filtering. Dee River water was filtered through 0.7 µm GF/F filters 48 hours prior to use in test solutions.

Two trials were conducted for each water type using the following treatment combinations: 0 mg/l, 10 mg/L and 20 mg/L Aldrich humic acid (AHA), and pH values of 3.5, 4, 4.5, and 5. The AHA used in this study was characterised by the following chemical features: residue on ignition 29.9%, carbon content 40.15%, hydrogen content 3.60%, and nitrogen content 0.92%. An additional treatment at pH 6 acted as the control for the soft water trial, whereas pH 7 was used as a control for the hard water and Dee River water trials. The pH was adjusted using HCl every 24 hrs throughout the trial, to keep pH drift to a minimum.

Toxicity tests were conducted over 96 hours in 400 mL plastic containers, each containing 200 mL of test solution and three shrimp. Each individual tested solution had three replicate containers giving n = 9 shrimp exposed to each treatment and a total of n = 135 shrimp in each trial. Organisms were checked every 24 hr for mortality and dead individuals were removed daily. The criteria for determining mortality were no movement and no reaction to gentle prodding. Organisms were not fed during the acclimation period and the trials as per ASTM (2007) guidelines. Test solutions were maintained using the static technique. Air was supplied to the containers continuously from an electric aerator. Lids were placed on top of the test chambers to stop contamination and the escape of test subjects. All trials, where possible, followed the recommendations made in reference [21].

During the experiment, pH (TPS 80A), conductivity (TPS LC84), oxygen (TPS WP-82Y) and ammonia levels (Aquarium Pharmaceuticals Inc., freshwater total ammonia test kit) were measured every 24 hrs. Temperature loggers (Model TG-4100, Tiny Tag) recorded temperature readings every hour. Disturbance to test organisms was minimal during water quality analyses. DOC measurements were conducted on river water, filtered with Sartorius 0.45 µm cellulose acetate filters, as provided by ALS analytical services and analysed after filtration using a Sievers 5310 CTOC Analyser 900 instrument. Samples for the determination of cations and anions were analysed using the automated potentiometric method for the determination of ions except for sulfate, which was estimated using the anion-cation balance method [22].

Mortality results for each test water from the two trials were pooled together and analysed using non-linear (three-parameter sigmoidal) regressions to determine LC_{50} values and their corresponding ninety-five percent confidence limits (SigmaPlot 11.0). Multiple line and scatter plots were prepared for each trial to show the differences in the proportion of organisms surviving between treatments.

3. RESULTS

3.1. Water Quality

Details of the composition of the three treatment waters are provided in Table 1. Key water quality parameters recorded during the trials are displayed in Table 2. The pH of treatment water showed some drift over the 24 hrs; however, this was kept to 0.4 units (Table 2). Oxygen levels were mostly above 80% saturation; temperatures were 21.5 ± 1°C and ammonia was low, with 1 ppm or less recorded for test waters during all trials (Table 2). Conductivity of treatments increased with decreases in pH (Table 2).

3.2. Ecotoxicity

Shrimp survival was 100% in almost all control treatments, except the 0 mg/L soft water treatment in trial one, and the 10 mg/L soft water treatment in trial 2, which had one death each (Figures 1 and 2). Meanwhile, 100% mortality of shrimp was always recorded in the 0 mg/L AHA treatment at ≤ pH 4.5 in hard water, at ≤ pH 4 in Dee River water and at pH 3.5 in soft water. Complete mortality of shrimp was also recorded in the 10 and 20 mg/L AHA treatments in all test waters at pH 3.5 and in the pH 4 solutions in hard water.

At pH 4, the addition of AHA to soft water was shown to increase shrimp survival by up to 33% and 44% at the 10 and 20 mg/L treatment levels, respectively (Figure 1). By comparison, at the same pH level, survival was improved by only 11% following the addition of 10mg/L AHA to Dee River water. The protective effect was markedly improved at pH 4.5, however, with the presence of AHA increasing, survivorship in Dee River water was observed by up to 34% (at 10 mg/L) and 38% (at 20 mg/L) (Figure 1). The best rates of improvement to shrimp survivorship were recorded for hard waters, where survival at pH 4.5 was increased by 56% in the 10 mg/L treatment AHA and by 44% in the 20 mg/L AHA treatment (Figure 1).

The 96-hour LC_{50} values calculated by non-linear regression varied across the different water types, and with AHA treatment level (Figures 2 and 3). For water without the addition of AHA LC_{50} values in hard water (4.95 ± 0.07) were slightly higher than Dee River water (4.74 ± 0.11) and substantially higher than soft water (4.27 ± 0.17) (Table 3). With the addition of AHA, LC_{50} values were substantially lower in both the artificially hard and Dee River waters (Table 3).

4. DISCUSSION

Increased toxicity of low pH to freshwater shrimp was shown to occur in the artificial hard water treatment. The reduced effect of the natural hard water (Dee River water) compared to artificial hard water in the 0 mg/L AHA treatments is likely due to the presence of DOC in the water. The Dee River water used in the experiments sourced from a natural river system as such still contained DOC (11 mg/L). This background DOC may have decreased the toxicity brought about by increased water hardness. Glover and Wood [23] have shown that increases in DOC can eliminate the adverse effects brought about by increased calcium on ion loss in *Daphnia magna*. The decreased toxicity of pH in soft water compared to hard water was surprising as it has been suggested that increased calcium levels associated with increased hardness decreases the adverse effects of low pH [18,19].

The consensus on the ameliorating effects of calcium to decreases in pH has come from studies on vertebrates such as fish and has been suggested to not apply to invertebrates [24]. Glover and Wood [24] showed that calcium offered no protection to ion loss in the invertebrate *Daphnia magna* with increases in calcium causing the inhibition of sodium uptake in this microcrustacean when exposed to pH 4. This inhibition of sodium influx caused by the increased calcium can potentially lead to mortality if whole body sodium contents are depleted [24-26]. The inhibition of sodium influx may reflect competition between sodium and calcium uptake at the sodium-proton exchanger, with calcium out-competing sodium for uptake [24,27,28]. Unlike the apical exchanger found in vertebrates such as fish, the sodium-proton exchanger in invertebrates exhibits strong calcium dependence, allowing it to transport calcium ions across the apical surface of the gills [27]. This ability helps invertebrates maintain calcium homeostasis, which is required for successful moulting [29]. The increased mortality of shrimp recorded in hard waters compared to soft waters throughout this study may be linked to the competition between calcium ions and sodium ions, leading to decreased sodium influx.

Table 1 Composition and water quality of test waters (mg/L).

	DOC	Hardness ($CaCO_3$)	Alkalinity ($CaCO_3$)	Ca	Mg	Na	K	Cl	SO_4
Dee River water	11	137	247	22	20	90	1.3	101	10
Artificial Hard water	0	120	107	25	14	52	4.8	80	<0.01
Artificial Soft water	0	32	1	3	6	14	3.1	26	<0.01

Table 2 Ranges of physicochemical variables recorded every 24 hrs during the 96 hr trial period for all trials.

Water type	pH Treatment	pH	Conductivity (µS/cm)	Oxygen (% sat)	Ammonia (ppm)
Soft	3.5	3.51 – 3.87	241 – 292	83 – 91	0.25 - 0.5
	4	3.98 – 4.39	182 – 214	83 – 89	0.25 - 1
	4.5	4.49 – 4.87	165 – 201	79 – 89	0.25 - 0.5
	5	5.02 – 5.42	152 – 185	82 – 90	0.25 - 1
	Control	6.00 – 6.35	143 – 159	78 - 91	0.25 - 0.5
Hard	3.5	3.50 – 3.66	1107 – 1166	81 – 88	0.5 - 1
	4	4.02 – 4.28	720 – 771	81 – 92	0.25 - 1
	4.5	4.52 – 4.88	596 – 646	81 – 89	0.25 - 0.5
	5	4.99 – 5.38	555 – 585	82 – 91	0.25 - 1
	Control	7.01 – 7.24	506 – 538	79 – 92	0.25 - 0.5
Dee River	3.5	3.51 – 3.67	1362 – 1435	81 – 86	0.5 - 1
	4	4.02 – 4.29	1112 – 1311	82 - 89	0.5 - 1
	4.5	4.49 – 4.87	1082 – 1218	81 - 87	0.5 - 1
	5	4.98 – 5.39	970 – 1083	82 - 90	0.5 - 1
	Control	7.18 – 7.55	821 – 931	81 - 89	0.5 - 1

n=30 readings

Table 3 LC_{50} values for each test water and each AHA treatment ± 1 STD.

	Hard water	Soft water	Dee River water
0 mg/L AHA	4.95 ± 0.07	4.27 ± 0.17	4.74 ± 0.11
10 mg/L AHA	4.47 ± 0.09	4.21 ± 0.14	4.55 ± 0.08
20 mg/L AHA	4.58 ± 0.12	4.18 ± 0.13	4.50 ± 0.06

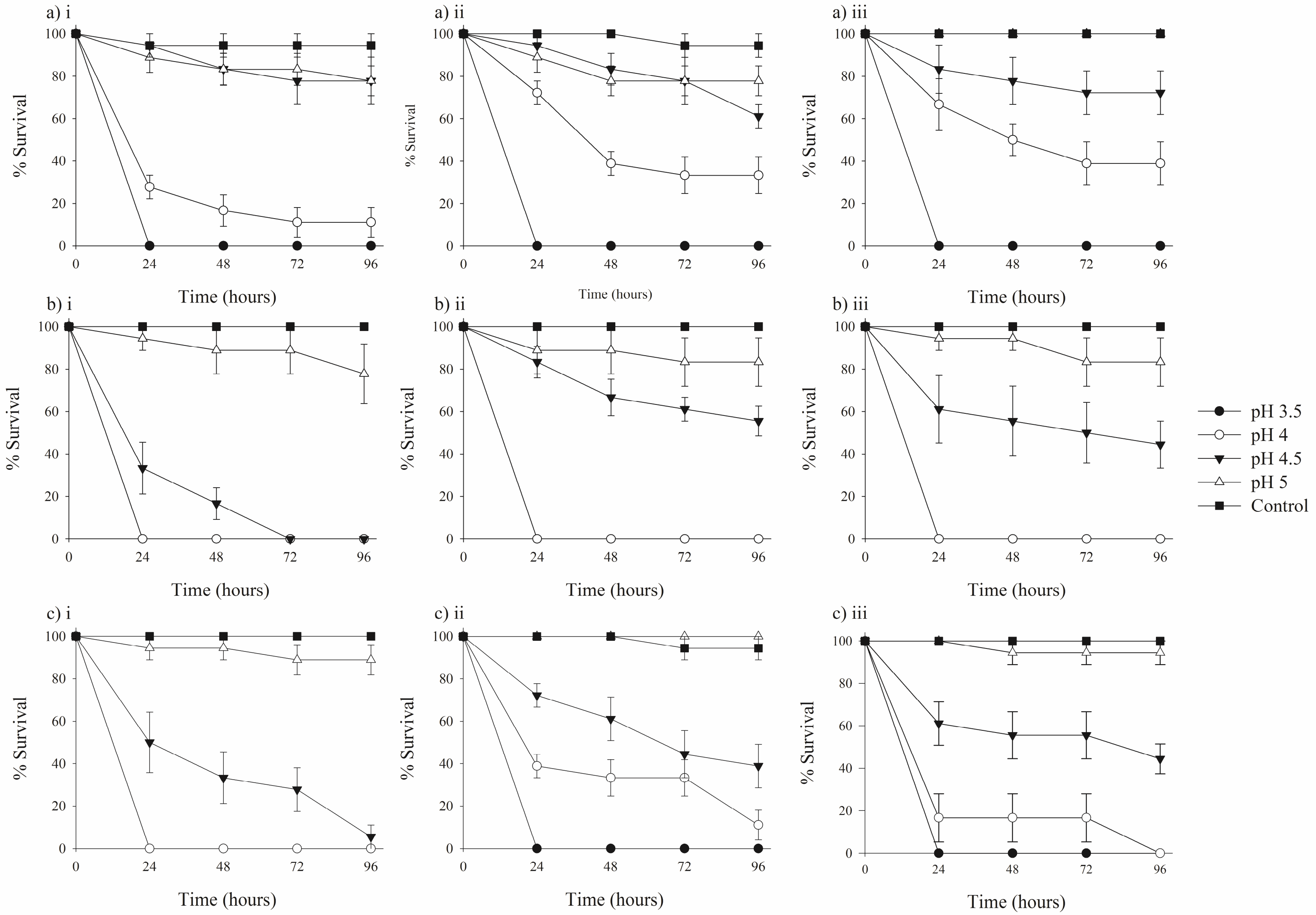

Figure 1 % Survival of shrimp over time in a) soft water; b) hard water; and c) Dee River water; where i = 0 mg/L AHA; ii = 10 mg/L AHA; and iii = 20 mg/L AHA. Each point is the mean of six replicates over two trials ± standard error (SE).

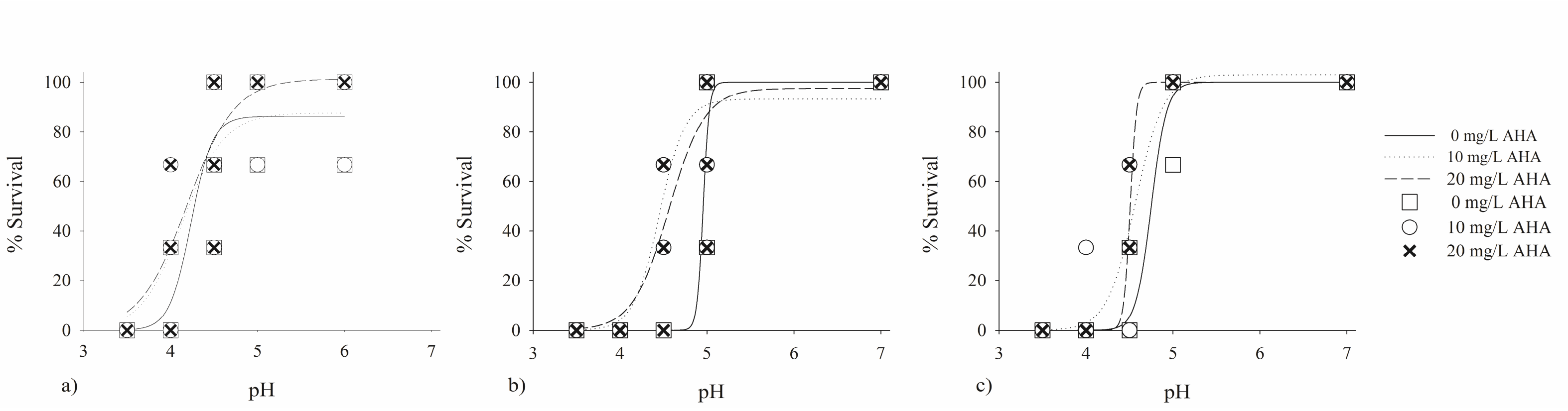

Figure 2 Concentration response plots for a) soft water, b) hard water and c) Dee River water; with 0, 10 or 20 mg/L AHA. Curves represent the non-linear regression (three-parameter sigmoidal) of pooled data from the two trials at 96 hrs.

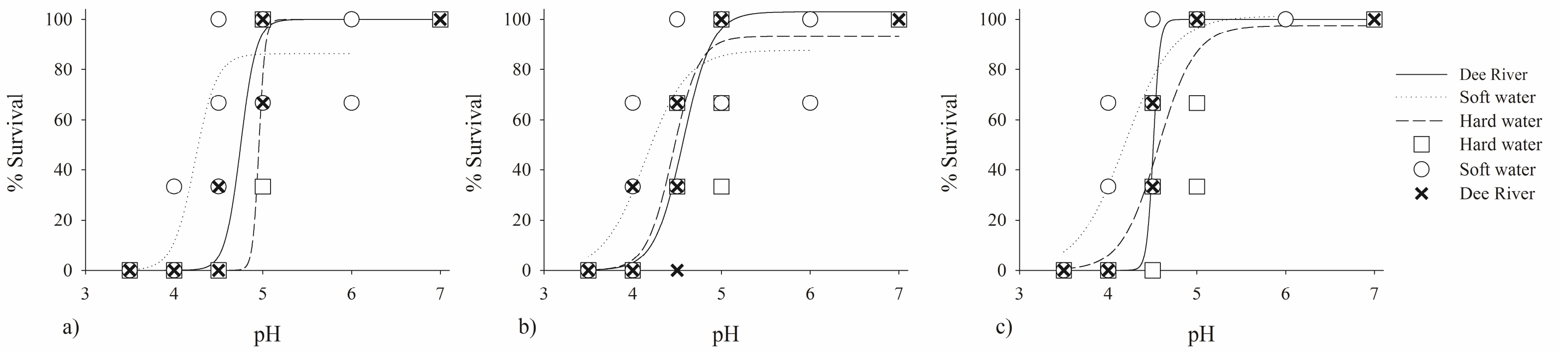

Figure 3 Comparison of concentration response plots for all water types at a) 0 mg/L AHA, b) 10 mg/L AHA and c) 20 mg/L AHA. Curves represent the non-linear regression (three-parameter sigmoidal) of pooled data from the two trials at 96 hrs.

This in turn may have led to the depletion of whole body sodium content, which eventuated in the mortality of the shrimp. The increased protection suggested to be offered by calcium may only apply to vertebrate aquatic organisms, with acidification of freshwaters high in calcium possibly having a greater effect on invertebrate populations than in soft freshwaters.

The addition of HS was shown to increase survivorship of *Caridina* sp. D to low pH irrespective of water chemistry (soft compared with hard water). These results provide support for the hypothesis that HS offers organisms protection against the detrimental effects of low pH in naturally acidic waterways and this ability appears to extend to other water types. However, it seems that the beneficial effects of HS on ameliorating the toxicity of pH is still somewhat limited by water chemistry, with HS treatments in hard water still recording higher toxicities than soft water without HS.

The ability of HS to increase survivorship to low pH shown in this study may be due to its ability to bind to the cells of gills and alter their permeability. Campbell et al. [30] found evidence that dissolved organic matter high in HS can bind to fish gill cells, changing the permeability of the cell membrane and thereby affecting the transport of molecules through the cell membrane. HS have also been shown to decrease the loss of ions such as sodium and chloride into the surrounding water at low pH [23,31]. Recent studies by Galvez et al. [32] and McGeer et al. [33] also show that HS can alter the transepithelial potential of gill epithelia and increase the Na^+, K^+-ATPase activity in the gills after long-term exposure.

HS may have also influenced survivorship by training the shrimp's chemical defence system to better cope with exposure to low pH [34]. HS exert a mild chemical stress on aquatic organisms by inducing biochemical defence systems such as heat shock proteins, molecular chaperones (stress proteins), and biotransformation enzymes [35]. By exerting a mild stress on the organism, it has been suggested that HS provide the organism with mechanisms to better cope with exposure to other environmental stressors such as pH [36]. Recently it has been shown that most cross-tolerances to environmental stressors are mediated by heat shock proteins [37,38] and that this response can occur rapidly [39]. In the case of HS, exposure to HS was able to alleviate the stress caused by increases in salinity, poor quality food and handling to aquatic organisms [34,36,40]. Pre-exposure of mayflies to high amounts of HS in naturally acidic streams may have led to their higher tolerance to acid mine drainage than mayflies sourced from other streams [41].

As AHA is a sodium salt, the addition of this compound to the treatment solutions may have increased sodium concentration in the 10 and 20 mg/L AHA treatments compared with the control (0 mg/L AHA). Sodium concentrations were not monitored within treatments during the trial period, so it is difficult to determine precisely what impact AHA addition may have had on the sodium concentrations. A study by Matsuo et al [42] reported that the addition of AHA to treatment water increased sodium concentrations by 38±1, 48±1, 67±1, and 116±5 µmol/L for the 5, 10, 20, and 40 mgC/L respectively. These values reflect the assumption that 40% of AHA is DOC. These values correspond with approximately 12.5 mg/L AHA, 25 mg/L AHA, 50 mg/L AHA and 100 mg/L AHA. If a similar calculation was applied to the current study, it suggests that the addition of AHA to the treatment concentrations would have increased sodium concentrations only marginally, for example, by <38 µmol/L Na^+ for the 10 mg/L AHA treatment and <48 µmol/L for 20 mg/L. Furthermore, the potential for elevated sodium concentrations in treatment waters containing AHA as opposed to control waters (0 mg/L AHA) in confounding the toxicological results is unlikely as effects of external sodium concentrations at below 48 µmol/L where shown by Glover et al. [23] previously to have little effect on sodium influx at pH 4 in the presence of AHA. Other studies investigating the influence of AHA on ion losses at low pH have not explored the possible implications of the increased external Na^+ ions brought about by the addition of AHA to treatments, with these studies suggesting that the ability of AHA to influence ion losses in aquatic organisms is likely due to its influence on cell membrane permeability [11,23,31].

Naturally acidic waterways high in HS have been shown to contain significantly higher diversity than waterways acidified by anthropogenic means [2,10,43]. This has been linked in the past to the adaptation of aquatic organisms to low pH in naturally acidic systems over evolutionary time [1,10,44]. The ability of HS to influence survival to low pH irrespective of water hardness, as shown by the results in this study, may also be a key factor. The bioavailability of heavy metals has also been linked to the differences between these two acidified aquatic environments [10,43], with heavy metals less bioavailable in naturally acidic systems due to the high amounts of HS present. Recent work has shown that addition of HS to water contaminated with acid mine drainage, (a common cause of anthropogenic acidified environments), increased survivorship of freshwater shrimp through complexation and precipitation of the heavy metals [45]. It is likely that decreases in pH will have a greater effect in systems with low levels of DOC due to the lack of buffering provided by HS and increased metal loads. This has important implications for the management of naturally acidic and anthropogenically acidified waterways. Holland et al. [3] suggested that decreases in the amount of HS in naturally acidic systems is likely to have drastic implications on their biodiversity and ecosystem functioning. Therefore, ensuring the continuous input of HS from surrounding vegetation into these systems is maintained is essential [3]. The addition of HS to anthropogenic streams may also provide a possible remediation strategy for these waterways as well as allowing for the possible recolonization of these systems from organisms commonly found in naturally acidic systems. The recolonization may occur naturally through such means as aerial deposition as waterways acidified from anthropogenic means are commonly found in close proximity to naturally acidic environments.

Given that AHA is terrestrial in nature, it may have different composition compared with HS extracted from freshwaters, and thus may elicit different protective effects in aquatic organisms. For example, a number of studies have shown quite distinct effects of AHA compared to natural aquatic HS isolates [11,23]. In fact, given the heterogeneous nature of HS in general, it is recommended that further research should use natural isolates for testing where possible, and particularly so if the studies are to be used to establish guideline values relating to the level of HS needed to maintain diversity within natural acidic waters. Notwithstanding this, the ability of AHA to increase survivorship of shrimp to acidification is a very important finding in the context of possible future remediation of anthropogenic acidified waters. Here, the likely sources of HS for remediation would be terrestrial in nature – for example, HS isolated from green waste and brown coal – thus, laboratory studies using HS (such as AHA) are very useful in guiding the most promising environmental sources.

5. CONCLUSIONS

This study has demonstrated the ability of HS to influence the toxicity of pH in both soft and hard waters. This has important implications for the management of naturally acidic waterways, as well as the treatment of waterways affected by anthropogenic acidification. As this study focussed on only acute toxicity, chronic studies on a range of different invertebrates are needed to explore the influences that water hardness and HS play in determining the toxicity of low pH at different water hardness and HS levels. Future studies on the impact of anthropogenic acidification on invertebrate communities should consider the combined effect of hardness, DOC and pH on taxa richness.

ACKNOWLEDGEMENTS

This study was supported by the Women's Equal Opportunity Postgraduate Research Award, the Centre for Environmental Management, and CQUniversity. The authors would like to acknowledge Dr Satish Choy for confirmation of shrimp identification, and Heather Smyth for help with water chemistry.

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AES 130226(1128)