Can the Biotic Ligand Model Predict Cu Toxicity Across a Range of pHs in Softwater-Acclimated Rainbow Trout?

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This study examined the effects of pH (5.0–8.5) on the toxicity of waterborne Cu to juvenile rainbow trout (Oncorhynchus mykiss) in soft water under flow-through conditions. Relationships between 96 h or 30 day Cu toxicity and 24 h lethal Cu accumulation on the gills (24 h LA50-Acute or 24 h LA50-Chronic) were examined in the context of predictions made using the biotic ligand model (BLM). Acute toxicity was relatively constant across pHs except for a 2- to 3-fold higher LC50 at pH 5.0. In the chronic exposure, the fish had similar tolerance to Cu from pH 5.0 to 8.0, but were 3- to 4-fold more tolerant at pH 8.5. This pattern was not captured by the current BLM which predicts that acute and chronic LC50 values should increase progressively from pH 5.0 to 8.5, with much greater values than those observed at the higher pH range. BLM-based water quality criteria would not be protective for trout at pH 8.0 or 8.5 in acute exposure to Cu in soft water. The measured 24 h LA50-Acute and LA50-Chronic at pH 8.5 were higher, and 24 h LA50-Chronic at pH 5.0 was lower than those at the other pHs. This study indicates that gill Cu bioaccumulation does not explain toxicity at high or low pH, and the BLM needs revision to adequately predict Cu toxicity to trout in soft water.

Introduction

Water chemistry parameters such as hardness, pH, alkalinity, and natural organic matter all influence the toxicity of Cu to freshwater organisms (1). Water pH may be particularly important in ion-poor waters that are susceptible to acidification in Scandinavia (2) and in the Canadian Shield region (3). In fathead minnows, Erickson et al. (1) demonstrated that the acute toxicity of Cu increased with a decrease in pH, as a result of the effects on speciation and complexation of Cu. However, results from studies with other fish species indicated a reverse pattern, which was thought to be mainly due to the competition between H+ and Cu2+ binding to the gill (4). In addition, Cu toxicity may not only depend on speciation and competition between ions in the water because H+ alone can have adverse effects on ionoregulation of fish (5). However, most of these pH effects on Cu toxicity have been observed in acute exposures only. Physiological compensations may (6) or may not occur in fish (7) when they are acclimated to both Cu and low pH.

Mortality is often associated with the gill metal burden in fish (8). The binding affinity and capacity of the gill for metals can be quantified and related to acute toxicity as demonstrated by the biotic ligand model (BLM) (9). Variation in sensitivity to metals within or among fish species may be explained by gill-binding characteristics and the 24 h LA50 (short-term accumulation in the gill that is associated with eventual 50% mortality in the exposure) (10). However recent studies have demonstrated that gill-binding characteristics can be modified by chronic exposure to waterborne metals, altered pH, water hardness, and dietary composition (11). For instance, H+ (12) and Ca2+ (13) can alter gill membrane permeability and ion transport, beyond just competing with metal cations for gill binding sites. The physiological impacts of these factors have not yet been incorporated into the BLM.

Among fish species, rainbow trout (Oncorhynchus mykiss) are well-known to be highly sensitive to Cu (14). The primary objective of this study was to examine the effects of pH on acute and chronic waterborne Cu toxicity (survival and biomass changes) in the softwater-acclimated rainbow trout in the context of the current BLM. Secondly, we investigated corresponding effects on whole body Cu and Na concentrations. To our knowledge, this is the first study to investigate the effects of pH on chronic Cu toxicity in freshwater fish. We attempted to explain the effects by the variation in Cu accumulation pattern (gill accumulation at 24 h and 30 day, and 24 h LA50-Acute and 24 h LA50-Chronic), and evaluated whether the current BLM adequately predicted the observed responses.

Materials and Methods

Experimental Fish and Softwater Acclimation. Juvenile rainbow trout, Oncorhynchus mykiss (2.5–3 g) obtained from the Humber Springs Trout Farm, Orangeville, ON, Canada were initially held in flowing dechlorinated City of Hamilton tap water (hard water) from Lake Ontario (hardness: 120 mg L⁻¹ as CaCO₃; Cu: 0.5–1 µg L⁻¹; pH: 7.8–8.0; temperature: 12 °C) in 500 L polyethylene tanks for 1 week. Tanks were aerated and subjected to a 12 h light: 12 h dark photoperiod. Fish were fed a 2% wet weight body ration daily. From the second week onward, the tap water flow rate was reduced gradually every 2 days, while the flow rate of ion-poor water from a reverse osmosis system was increased gradually, until the resulting soft water had the following ionic composition: Na⁺: 0.11 mM; Cl⁻: 0.14 mM; Ca²⁺: 0.10 mM; Mg²⁺: 0.04 mM; hardness: 14–22 mg L⁻¹ as CaCO₃; alkalinity: 32 mg L⁻¹ as CaCO₃; DOC: 1.5 mg L⁻¹; Cu: 0.5–1 µg L⁻¹; pH: 7.0. Fish were acclimated to the resulting soft water, typical of the Canadian Shield Lakes, for an additional week prior to their use in experiments.

Control of Water pH. Fish were transferred to individual 20 L flow-through aerated tanks for Cu exposure. All experimental tanks were connected to a head tank that supplied a combination of hard and ion-poor water. To maintain the pH, a pH electrode was immersed in the head tank and transferred signals to a pH titrator (TTT80 titrator, Bach-Simpson Ltd.) through a pH meter. A pH end point valve was set in the pH titrator where it automatically triggered a valve to open; this would allow 1 N HCl (prepared from ACS reagent grade 37% HCl) or 1 N NaOH (prepared from ACS reagent grade NaOH pellets) into the head tank to adjust the...
pH. Every day, the pH of water in the experimental tanks was checked using a portable pH meter and the pH end point was adjusted accordingly to maintain the pH. The tested pH values were nominally 5.0, 6.0, 7.0 (baseline), 8.0, and 8.5. The lower end of the pHs tested was representative of acidified lakes in Ontario, Canada (15).

Acute Exposure (96 h). Fish in the acclimation tanks were not fed for 1 day to clear their gut of faeces that might adsorb Cu from the water. Seventeen fish were then transferred to each of the six experimental tanks with soft water at baseline pH. Except for the baseline pH experiments, the fish were acclimated to a gradual change of pH for 1 day in the experimental tanks. On the next day (0 h), Cu stock solution (as CuSO₄·5H₂O salt) was added to the tanks to achieve a range of Cu concentrations from Maritote bass (Figure 1B), but were 3-4-fold more tolerant at pH 8.5 (LC50, LC20, and LC10; Table 1).

The ratios of 96 h LC50/30 day LC20 (ACR) were 3.9, 1.1, 1.8, 1.0, and 0.4 at pH 5.0, 6.0, 7.0, 8.0, and 8.5, respectively. In general, the concentrations of Cu that killed 50% of trout were similar between the acute exposures and chronic exposures, except at the low and high pHs (Table 1). This may imply that mainly acute toxic actions of Cu occur in the rainbow trout, such that longer exposure may not increase toxicity, in agreement with the results of DeSchampelhaere and Janssen (17) for the toxicity of Zn in trout. On the other hand, the provision of food in the chronic exposure may play an important protective role. D’Cruz and Wood (18) and D’Cruz et al. (19) demonstrated that trout provided with food with sufficient salt content can compensate for the increased energy expenditures or difficulties in maintaining ion balance associated with low environmental pH stress. In addition, metal detoxification mechanisms (e.g., metallothionein) may be induced in rainbow trout after short-term metal exposure, thereby conferring greater resistance during chronic exposures (20). DOC released from food may also reduce Cu bioavailability to the trout (9), however, this was unlikely in our system which was flow-through and DOC concentrations were comparable between the acute and chronic exposures (SI Table S1, S2). In general, our study demonstrates that trout provided with food is expected to have a higher LC50 than trout provided with food that is saltless, indicating that trout provided with food is more tolerant of Cu than trout provided with food that is saltless.

Results and Discussion

Acute and Chronic LC Values. In the acute exposure, 96 h LC50, LC20, and LC10 values were relatively constant across pHs (Table 1, Figure 1A), except for the 2-3-fold higher LC50 at pH 5.0; the LC20 and LC10 at pH 5.0 were not significantly elevated. In the chronic exposure, the pattern was different. Fish exhibited a similar tolerance to Cu from pH 5.0 to 8.0 (Figure 1B), but were 3-4-fold more tolerant at pH 8.5 (LC50, LC20, and LC10; Table 1).
increases, the fraction of copper carbonate and copper pH in fathead minnows. According to the BLM, as pH increases at higher pH, this may result in very significant difference of group from others.

Moreover, all of these would be offset by the reduced competition of \( H^+ \) and \( Cu^{2+} \) for binding to the gill, thus toxicity, on the basis of free Cu ion, can be increased at higher pH. This study also demonstrated an apparent trend of decreasing free Cu ion LC50 with increased pH. This finding agrees with Borgmann (22) and French and Hunt (23), who suggested Cu complexes may result in some toxicity at higher pH, and indeed the BLM contains a modification for CuOH+ binding to the gills and resulting toxicity to account for this effect.

From a physiological perspective, the independence of LC values from pH in this study may be explained by the buffering of pH in the brachial microenvironment, such that it is much more constant next to the gills than in the bulk water (24, 25). In rainbow trout, acidic soft water is rendered more basic, whereas basic soft water is rendered more acidic, as it passes over the gills (25), reflecting the movements of \( NH_3, CO_2, \) and \( HCO_3^- \) across the gill surface during respiration (26). Therefore, the change of gill microenvironment pH is considerably smaller compared to the pH change in the surrounding water. This may result in very similar 96 h LC50 values across pHs, when expressed as dissolved Cu or total Cu, except in the very acidic and alkaline conditions. However when expressed as free Cu ion based on bulk water pH, the 96 h LC50 values would appear to vary greatly (Figure 1C).

**Acute and Chronic LC Values Predicted by the BLM.**

Except at pH 5.0, the Cu 96 h LC50 and 30 day LC20 values predicted by the BLM were all greater than measured values, and increased markedly with pH, such that the discrepancy became progressively larger at higher pHs (Table 1). Using the traditional criterion of ±2 x 2 for acceptable error between predicted and measured values, the BLM overestimated the 96 h LC50 at all pH values except for pH 5.0, as well as the 30 day LC20 except at pH 5.0 and 6.0. The U.S. EPA water quality criteria predicted by the BLM for Cu, both the criterion maximum concentration (CMC, recommended criterion for acute protection) and the criterion continuous concentration (CCC, recommended criterion for chronic protection) were also greater at higher pHs (Table 2). At pH 8.0 and 8.5, the CMC is similar to or higher than the measured 96 h LC50, and therefore is clearly not protective. However, it should be noted that softwater lakes with high pH are not common, at least in Ontario (27). The CCC is below the measured 30 day values.

### TABLE 1. Measured Cu LC values in Rainbow Trout in Different Water pHs, in Comparison to The BLM-Predicted Values, and BLM-Based Water Quality Criteria (CMC and CCC) for Rainbow Trout in Soft Water Conditions

<table>
<thead>
<tr>
<th>experiment</th>
<th>LC50 measured</th>
<th>predicted</th>
<th>LC20 measured</th>
<th>predicted</th>
<th>LC10 measured</th>
<th>predicted</th>
<th>CMC</th>
<th>CCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>acute</td>
<td>pH 5</td>
<td>19.4 (10.1–28.7)*</td>
<td>11.9</td>
<td>11.5 (2.9–17.5)</td>
<td>NA</td>
<td>8.8 (1.4–14.3)</td>
<td>NA</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>pH 6</td>
<td>5.9 (4.6–7.2)</td>
<td>34.9</td>
<td>3.3 (2.0–4.4)</td>
<td>NA</td>
<td>2.5 (1.3–3.4)</td>
<td>NA</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>pH 7</td>
<td>5.2 (5.7–12.7)</td>
<td>73.6</td>
<td>4.7 (1.6–7.8)</td>
<td>NA</td>
<td>5.2 (4.3–6.0)</td>
<td>NA</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>pH 8</td>
<td>8.5 (7.1–10.3)</td>
<td>112.0</td>
<td>5.6 (4.1–6.8)</td>
<td>NA</td>
<td>4.5 (3.0–5.6)</td>
<td>NA</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>pH 8.5</td>
<td>6.7 (5.8–7.5)</td>
<td>172.9</td>
<td>4.8 (3.5–5.5)</td>
<td>NA</td>
<td>4.0 (2.6–4.8)</td>
<td>NA</td>
<td>15.1</td>
</tr>
<tr>
<td>chronic</td>
<td>pH 5</td>
<td>8.1 (4.6–10.9)</td>
<td>NA</td>
<td>5.0 (1.7–7.3)</td>
<td>3.4</td>
<td>3.8 (1.0–6.0)</td>
<td>NA</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>pH 6</td>
<td>7.4 (6.4–9.6)</td>
<td>NA</td>
<td>5.5 (4.5–6.3)</td>
<td>10.2</td>
<td>4.7 (3.4–5.4)</td>
<td>NA</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>pH 7</td>
<td>8.2 (7.3–9.1)</td>
<td>NA</td>
<td>5.2 (4.3–6.0)</td>
<td>22.7</td>
<td>3.9 (3.0–4.9)</td>
<td>NA</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>pH 8</td>
<td>9.3 (6.6–10.6)</td>
<td>NA</td>
<td>8.2 (1.9–9.1)</td>
<td>36.9</td>
<td>7.6 (4.1–8.7)</td>
<td>NA</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>pH 8.5</td>
<td>22.4 (16.5–27.5)*</td>
<td>NA</td>
<td>18.1 (10.3–22.1)*</td>
<td>52.1</td>
<td>16.1 (7.9–20.1)*</td>
<td>NA</td>
<td>10.3</td>
</tr>
</tbody>
</table>

* CMC: criterion maximum concentration for acute exposure; CCC: criterion continuous concentration for chronic exposure. NA: Not applicable. All concentrations are expressed in \( \mu g L^{-1} \) with 95% confidence intervals. Asterisk indicates significant difference of group from others.

![Graph](https://example.com/graph.png)

**FIGURE 1.** Rainbow trout Cu LC values (±95% CI) in (A) 96 h (acute) and (B) 30 day (chronic) exposures at different water pHs. In (C), the effect of pH on Cu 96 h LC50 is expressed on the basis of total Cu and free Cu ion; note the logarithmic scale. Significant differences of LC values from other pH values are marked by an asterisk. A linear regression line was fitted to the free Cu ion LC50 against pH data, at \( P < 0.05 \).
at high pH values, and is therefore protective in chronic exposure to Cu, but the margin of protection is relatively small. The criteria do protect the trout well against Cu toxicity at lower pHs, but they appear potentially overprotective for trout (CMC or CCC: 0.03 – 0.5 µg L\(^{-1}\) vs LC\(_{50}\): 5.0 – 11.5 µg L\(^{-1}\) at pH 5.0) and not environmentally relevant because Cu concentrations in pristine soft water (0.2 – 1.5 µg L\(^{-1}\)) often exceeds these criteria concentrations. However, the criteria will be higher and justified in water with higher DOC (DOC in Ontario lakes: 1.5 – 13 mg C L\(^{-1}\)) (27). When the criteria are set based on water hardness only (14 – 22 mg L\(^{-1}\) as CaCO\(_3\) in our study), the U.S. EPA has a general CMC of 2.1 µg L\(^{-1}\) and CCC of 1.7 µg L\(^{-1}\), whereas the Canadian guideline (chronic only) of Canadian Council of Ministers of the Environment guideline (30) is 2 µg L\(^{-1}\). This demonstrates that the hardness-based guideline protects the trout from Cu toxicity in basic soft water. One explanation for overestimation of LC\(_{50}\) or LC\(_{20}\) values by the BLM may be a lack of equilibration of DOC and Cu in the flow-through water (residence time: 80 min). Another may be the calibration data sets from China for trout which are used in the current data. Coefficients of determination (r\(^2\)) for the significant regression lines are 0.96, 0.71, 0.86, 0.86, 0.84, and 0.94, 0.87, 0.99, 0.88, 0.95 for pH 5.0, 6.0, 7.0, 8.0, and 8.5 in the acute test and chronic tests, respectively. X-axis scale for the acute test does not include the highest Cu concentration (46.0 µg L\(^{-1}\)) used for pH 5.0, so the regression line extends beyond the range shown.

**TABLE 2. Lethal Accumulations of Cu at 24 h (24-h LA\(_{50}\)-Acute and 24 h LA\(_{50}\)-Chronic in gills of Rainbow Trout for Predicting Mortality of Acute (96 h) and Chronic (30 day) Exposures**

<table>
<thead>
<tr>
<th>pH</th>
<th>24-h LA(_{50})-Acute (96 h)</th>
<th>24-h LA(_{50})-Chronic (30 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>8.4 (5.9 – 11.9)</td>
<td>0.9 (0.7 – 1.3)*</td>
</tr>
<tr>
<td>6.0</td>
<td>5.1 (2.4 – 10.9)</td>
<td>11.1 (8.2 – 15.1)</td>
</tr>
<tr>
<td>7.0</td>
<td>9.7 (4.5 – 12.6)</td>
<td>6.5 (2.6 – 12.7)</td>
</tr>
<tr>
<td>8.0</td>
<td>8.1 (7.2 – 9.1)</td>
<td>6.7 (4.9 – 9.2)</td>
</tr>
<tr>
<td>8.5</td>
<td>49.2 (30.3 – 79.8)*</td>
<td>7.94</td>
</tr>
</tbody>
</table>

*Data are expressed in nmol g\(^{-1}\) wet wt. with 95% confidence intervals. Due to the low mortality of fish in pH 8.5 chronic Cu exposure, the corresponding 24 h LA\(_{50}\)-Chronic was only an estimate and no 95% confidence limits could be reported. Asterisk indicates significant difference of group from others. No statistical test was run for pH 8.5 (chronic).*

**FIGURE 2. Gill Cu accumulation (means ±1 SEM) at 24 h of the acute exposures (A) and 30 d in the chronic exposures (B) in rainbow trout at different pHs. Coefficients of determination (r\(^2\)) for the significant regression lines are 0.96, 0.71, 0.86, 0.86, 0.84, and 0.94, 0.87, 0.99, 0.88, 0.95 for pH 5.0, 6.0, 7.0, 8.0, and 8.5 in the acute test and chronic tests, respectively. X-axis scale for the acute test does not include the highest Cu concentration (46.0 µg L\(^{-1}\)) used for pH 5.0, so the regression line extends beyond the range shown.**

**Gill Cu Accumulation and LA\(_{50}\) Values.** Positive linear relationships between 24 h gill Cu accumulation and waterborne concentrations were observed in the acute exposures (Figure 2A). Relationships of gill Cu accumulations versus dissolved exposure concentrations were comparable at pHs ranging from 5.0 to 8.0 (slopes: 0.06 – 0.12 µg g\(^{-1}\) well wt. per µg L\(^{-1}\) but greater at pH 8.5 (slope = 0.69). At low pH, rainbow trout were particularly tolerant to Cu in the acute exposure, similar to results of previous reports (28, 29). Higher concentrations of H\(^+\) at low pH may outcompete Cu\(^{2+}\) for binding to the fish gill (21). However, this was not supported by the pattern of gill Cu accumulation as a comparable amount of Cu was bound to the gill from pH 5.0 to 8.0. Indeed, fish may secrete more mucus in acidic conditions (24, 30). Mucus can serve as an ion exchange system (31, 32) on the gill epithelium, providing protection by binding toxic metals and removing them when it is sloughed off (33, 34). Therefore, mucus secretion may play an important role in reducing acute Cu toxicity to the trout at pH 5.0. However, trout were more sensitive to Cu in the chronic versus the acute exposure at pH 5.0. This suggests that mucus secretion may not be sufficient to protect against Cu in the longer term.

Similarly, in the chronic exposure, positive relationships were observed between 30 days gill Cu accumulation and dissolved Cu concentrations (Figure 2B), but quadratic or sigmoidal relationships provided improved fits at pH 7.0 – 8.5. In general, gill Cu accumulations were higher at 30 days than at 24 h (Figure 2).

The constant 24 h LA\(_{50}\)-Acute across most pHs (Table 2) and the 10-fold higher 24 h LA\(_{50}\)-Acute at pH 8.5 corresponded to the 24 h gill accumulation patterns (Figure 2A). The 24 h LA\(_{50}\)-Chronic Values were comparable between pH 6.0 and 8.0 (and similar to 24 h LA\(_{50}\)-Acute Values) but significantly lower at pH 5.0 (unlike the acute data), and higher at pH 8.5 (similar to the acute data). Due to the different concentration ranges of Cu tested in the acute (highest: 9.9 µg L\(^{-1}\)) and chronic (highest: 32.3 µg L\(^{-1}\)) exposures at pH 8.5, the corresponding 24 h LA\(_{50}\)-Chronic could only be estimated from the normalized gill Cu binding in the fish exposed to the highest Cu concentration in the acute test and no 95% confidence limits could be reported. The 24 h LA\(_{50}\), in our study was in agreement with MacRae et al. (8) in rainbow trout (10 nmol g\(^{-1}\)) and by calculations (9, 21) based on the data of Playle et al. (33, 36) and Erickson et al. (11) in fathead minnows (12 nmol g\(^{-1}\)). According to a fundamental tenet of the BLM, the 24 h LA\(_{50}\) should be independent of water chemistry, therefore the 24 h
gill metal accumulation has been widely used to indicate bioavailability and toxicity of metals to fish in acute exposures (10, 37). However, in the present study, this concept appeared to be valid in the pH range of 6.0–8.0, but broke down at low (pH 5.0) or high pH (pH 8.5), with opposite discrepancies observed at pH extremes in the acute versus chronic exposures. Thus, at pH 5.0, 24 h $L_{A(20)}$-Acute was “normal” but 96 h $L_{A(20)}$ was high, whereas 24 h $L_{A(20)}$-Chronic was low, while 30 day $L_{A(50)}$ was “normal.” Conversely, at pH 8.5, the 24 h $L_{A(20)}$-Acute was high but the 96 h $L_{A(20)}$ was “normal”, while both the 24 h $L_{A(20)}$-Chronic and 30 day $L_{A(50)}$ were high. The reasons outlined above (mucus at low pH, CuOH$^+$ binding at high pH, pH buffering in the gill microenvironment) may provide a partial explanation for these discrepancies. Measuring Cu truly taken up by the gill, apart from that bound to mucus, may improve the relationship between accumulation and associated biological effects in low pH. Additionally, based on these results, there may be a need to reassess the binding constants of the gill for $H^+$ and for $Cu^{2+}$ at pH extremes, so as to improve BLM predictions in soft water.

Furthermore, it is questionable whether 24 h gill Cu accumulation can predict responses up to 30 day, given the time-dependent differences observed. The increase of gill Cu burden with time at most pH values and the change in gill accumulation pattern (linear to sigmoidal/exponential relationship) at a higher pH value in the chronic relative to the acute exposure provides additional evidence for alteration in gill-binding characteristics with long-term exposure to waterborne Cu. This agrees with Kamunde et al. (38) and Taylor et al. (19) who reported an increase of low-affinity, high-capacity Cu binding sites on the gill of rainbow trout after chronic exposure to Cu. Yellow perch (Perca flavescens) from a metal-contaminated lake also had higher Cu binding to the gill compared to those from a reference lake (39).

**Biomass.** Fish biomass was significantly reduced by chronic Cu exposure with effects similar between pH 5.0 and 8.5. The similar EC and LC values (SI Table S3, Table 1) indirectly provide evidence that Cu did not affect growth of fish which survived through the entire exposure period.

**Whole Body Na and Cu Concentrations.** Whole body Na concentration was reduced by Cu in the short term exposure and these effects were pH-dependent (SI Figure S1). Cu accumulation pattern in different exposure periods was different (SI Figure S2), but there were no clear patterns of pH effects. See SI for more details.

**Acknowledgments**

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**Supporting Information Available**

Methods for water chemistry and tissue analyses; Measured water chemistry values (Table S1, S2); Results and discussion on biomass (Table S3); Whole body Na (Figure S1) and Cu concentrations (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

**Literature Cited**

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