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Interactive effects of phosphorus and copper on *Hyalella azteca* via periphyton in aquatic ecosystems

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ABSTRACT

This research examined the interaction between dissolved copper and phosphorus, with respect to their effects on the freshwater amphipod *Hyalella azteca* feeding on periphyton. Field-collected periphyton communities were exposed to different nutrient and metal conditions in indoor recirculating streams. *H. azteca* were then exposed to water and periphyton from these streams. There was rapid Cu accumulation by periphyton but the total Cu concentration of periphyton was not directly related to dissolved P. In terms of *H. azteca* growth, an interactive effect was found between Cu and P as growth was reduced more than expected in the low Cu-high P treatment. Our data suggest that eutrophic conditions result in greater Cu toxicity to benthic macroinvertebrates at lower metal concentrations, likely due to higher assimilation efficiency of dietary Cu from periphyton incubated under eutrophic conditions. These results imply that non-additive interactions between multiple stressors may cause ecosystem effects as detected in standard laboratory bioassays conducted under controlled conditions.

1. Introduction

Aquatic ecosystems are rarely disturbed by a single type of stressor (Ivorra et al., 2002), and the impact of pollutants may interact with various biotic and abiotic stressors (Heugens et al., 2001). Thus, considering multiple-stressor scenarios would be a critical component in deriving risk assessments of real aquatic condition. Metal pollution in aquatic ecosystems from multiple sources such as urban wastewater, industrial and mine effluents (Nriagu, 1979) are frequently associated with eutrophic conditions (Lopez-Flores et al., 2003); although both problems have been extensively investigated, traditionally they have been treated as separate stressors. To better understand the impact of toxic metals in waterbodies with varying nutrient conditions, it is of great importance to elucidate the interaction between metal and nutrients with respect to their effects on biota.

An emerging approach for detecting the effects of aquatic toxicants (e.g., metals) is to examine natural periphyton communities, also called phototrophic biofilms (Sabater et al., 2007). Periphyton grows at the interface between overlying water and sediments, thus providing an integrated representation of the accumulation of toxicants in the aquatic environment (Lowe and Pan, 1996; Newman and McIntosh, 1989). Because of its sorptive nature coupled with large surface area (Hill et al., 2010), periphyton can take up significant quantities of heavy metal from water or sediments, producing an internal concentration often thousands of times greater than that of their surrounding environment (Genter, 1996; Hill et al., 1996).

Uptake of dissolved metal by periphyton can lead to decreased biomass and altered taxonomic composition (Guasch et al., 2002; Roussel et al., 2007; Serra and Guasch, 2009). Recent studies have found that under eutrophic conditions, periphyton communities could be more tolerant to metals (Serra et al., 2010). This greater metal tolerance led to less reduction in periphyton biomass but little information is available on how metal tolerance of periphyton in the presence of nutrients affects higher trophic-level organisms like benthic macroinvertebrates or fish.

This study aimed to evaluate the influence of nutrients on periphyton-mediated metal toxicity to stream macroinvertebrates. We focused on copper (Cu) and phosphorus (P) which are commonly found together in fluvial systems draining industrial and urban watersheds (Twiss and Nalewajko, 1992). We hypothesized that periphyton grown under higher eutrophic condition will contain relatively more Cu, which in turn poses a greater risk of dietary Cu exposure to consumers of periphyton. To test this hypothesis, we conducted experiments using indoor artificial streams to simulate environmentally realistic Cu exposure on periphyton and a potential grazer *Hyalella azteca*.

2. Material and methods

General study design: To detect interactive effects of phosphorus and copper on *H. azteca* via periphyton, this study was conducted in two tiers (Fig. 1): (1) field-collected periphyton communities were exposed to different nutrient and metal

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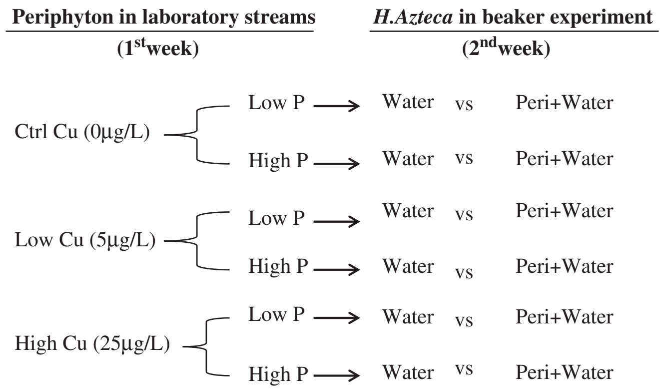

Fig. 1. Scheme of the experimental design. Cu-media refers to the nominal copper concentrations in water during one-week laboratory stream experiment. P-media refers to the nominal phosphorus conditions in water. Low and high P represent nominal dissolved phosphorus concentrations 50 µg/L and 250µg/L, respectively. Beaker experiments on *H. azteca* include two treatments (water and peri+water) for each Cu–P combination in order to distinguish the toxicity of dietary exposure through periphyton. Each Cu–P combination has 5 replicate beakers with 10 *H. azteca* in each beaker.

conditions in indoor recirculating streams; (2) *H. azteca* in beakers were then exposed to water and periphyton from these streams.

2.1. Waterborne Cu and P exposure to periphyton in laboratory streams

54 unglazed tiles (26 cm^2) within a plastic-coated basket were placed in the raceway of Saline Fisheries Research Station from August to October, 2010 to allow homogeneous colonization of a natural periphyton community. This fisheries research station is a freshwater field station of the Institute for Fisheries Research in Saline, Michigan. Limited human disturbance, sufficient natural light and nutrients with unpolluted water quality make it an excellent colonization site. After colonization, the tiles were transported to the lab and 9 tiles were randomly distributed into each stream (oval raceway: OD-30 cm, ID-10 cm, width=79 cm, depth=10 cm). Each of 6 streams included single combinations of dissolved Cu (3 treatments) and P (2 treatments) in reconstituted water containing some micronutrients (alkalinity=56 mg/L, hardness=92 mg/L, pH=7.7, conductivity=375 µs/cm) (EPA, 2000). To ensure sufficient amount of periphyton for feeding experiment to investigate the effects of Cu and P on *H. azteca*, we referred to former research by Roussel et al. (2007) and chose Cu exposure levels (control 0 µg/L, low Cu 5 µg/L, and high Cu 25 µg/L) to attain no or lowest adverse effect on biomass of periphyton.

For P, two concentrations (50 and 250 PO_4–P µg/L) were used to mimic mesotrophic and eutrophic conditions (Dodds et al., 1998). During the week-long exposure, water was continuously recirculated in the streams and reconstituted water was added daily to compensate for evaporation. On day 3, Cu and phosphate was added to maintain concentrations at treatment levels. During the experiment, illumination was provided by fluorescent grow lights (approximately 2000 lx, Hydrofarm) with a 16:8 h light:dark cycle. Light intensity, temperature, pH, dissolved oxygen (DO) and conductivity were measured daily. Every three days, water samples were filtered (0.7 µm pore size) for determination of dissolved Cu (ICP-OES, Optima 4300DV, PerkinElmer) and soluble reactive phosphorus (ascorbic acid colorimetric method) (Eaton and Franson, 2005).

Two colonized tiles from each stream were randomly sampled both 2 h and 7 d after exposure. Periphyton scraped from two tiles was homogenized and collected onto a preweighed glass fiber filters (pore size 0.7 µm). Biomass of periphyton was measured as both chlorophyll *a* (chl*a*) and ash-free dry mass (AFDM) (Steinman et al., 2007). Chl *a* was extracted from each filter in 20 ml of 90% ethanol in 78 °C water bath for five minutes (Biggs and Kilroy, 2000) and the concentration was then read by a fluorometer (Turner Designs TD-700). To measure Cu concentrations in periphyton filters containing dry periphyton were microwave digested (CEM MARS 5) with 4 ml of concentrated nitric acid and 1 ml of hydrochloric acid (EPA, 1996; Serra et al., 2009) and analyzed by ICP-OES.

2.2. Beaker experiment for waterborne and dietary Cu exposure to H. azteca

After exposing the periphyton for one week, water and colonized tiles were transported from each stream to 500 ml beakers for 7-day toxicity tests with 7–14 days old *H. azteca* laboratory-cultured as described in EPA standard methods (EPA, 2000). The mean body size of these 7–14 days old *H. azteca* was 2.75 ± 0.53 mm at the beginning of test. Two types of treatments for each stream were designed to separate waterborne exposure and dietary exposure. Water treatments (water) exposed *H. azteca* to stream water and one uncolonized tile while the combined treatments (peri+water) exposed *H. azteca* to stream water and one periphyton colonized tile (Fig. 1). Each treatment has 5 replicate beakers with water and one (un)colonized tile from the same stream and10 *H. azteca* in each beaker. The dietary and water-only exposures began at the same time among all treatments and lasted for one week. During the exposure, we fed *H. azteca* no other food to be certain that periphyton was the only dietary source. At the end of the beaker experiment, surviving *H. azteca* were counted and preserved in 70% ethanol (Hauer and Resh, 2006) for measurement of growth as the increase in body length. Individual *H. azteca* were photographed at 5× magnification and measured body length along the curve of the dorsal surface (±0.05 mm) (EPA, 2000) using ImageJ 1.43 u (National Institutes of Health).

2.3. Data analysis

- Periphyton: The ratio of AFDM to chl*a* of periphyton, called autotrophic index (AI), is calculated to investigate changes in the ratio of autotrophic to heterotrophic community structure and higher values indicate more heterotrophic dominance in periphyton community (Azim et al., 2005). Due to lacking stream treatment replicates for exposure to periphyton, statistics on chl*a*, AFDM, and Cu content of periphyton were not possible.
- *H. azteca*: The crude feeding rates of grazers in each replicate beaker in peri+water treatments are estimated by dividing changes in total dry mass of periphyton communities pre- and post-grazing by the length of exposure and number of *H.* azteca, and pre-grazing dry mass was represented by the final dry mass of periphyton after being incubated for 7 days in streams. Comparisons of *H. azteca* feeding rates, survival and growth among Cu and P treatments were made using two-way ANOVA tests followed by Tukey's post hoc test. All statistical analyses were performed using R version 2.12.1 (R Development Core Team 2010) and in all cases, $\alpha=0.05$ was chosen to interpret the significance of the effects and differences among treatments. No violations of normality or equal variance were found and transformations were unnecessary. When the Cu concentration of acid-digested solutions was below ICP-OES detection, the concentration was set at the detection limit (1 µg/L) for calculations and statistics.

3. Results

3.1. Waterborne Cu and P exposure to periphyton in laboratory streams

Most of the physical and chemical parameters of stream water did not show great differences among the six streams. The stream water was circum-neutral (pH=7.96 ± 0.07) and alkalinity around 80 mg/L $CaCO_3$. A gradual increase in conductivity and hardness were observed during the exposure period, likely due to evaporation and daily addition of reconstituted water. Three days after spiking, the dissolved Cu concentration decreased to less than 1.8 µg/L. Elevated (though variable) Cu concentrations within periphyton just 2 h post exposure were observed, implying rapid absorption of Cu by periphyton (Fig. 2A). After the 7-day exposure, periphyton Cu concentrations were much greater in the high Cu treatment compared to low Cu and control treatments, with a trend of slightly lower Cu in high P treatments (Fig. 2B).

After the 7-day exposure, both chl*a* and AFDM tended to be higher in the high P treatment than in the low P treatment at each Cu level (Table 1), which suggested greater food availability in high P treatments in the subsequent beaker experiment. Through time, both chl*a* and AFDM tended to decrease and AI (AFDM/Chl*a*) increased thereby periphyton community shifting to heterotrophy in all treatments. Compared with low P treatments, the community shifted in a less extent to dominance by heterotrophy in high P treatment under all Cu exposure levels and controls (Table 1). Periphyton in the low Cu-high P treatment has noticeably least shift in AI and highest autotrophic biomass among all treatments (Table 1).

3.2. Beaker experiment for waterborne and dietary Cu exposure to H. azteca

After one-week exposure, *H. azteca* survival was fairly high (>70%) and not significantly different among all treatments for either peri+water or water exposures ($p>0.05$). For *H. azteca*

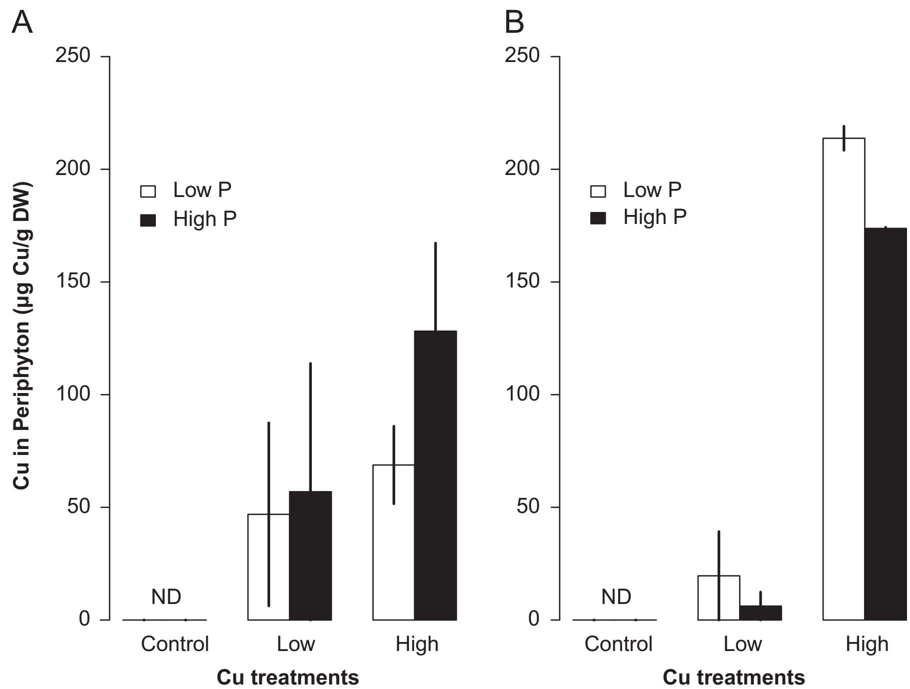

Fig. 2. Biomass-specific total Cu concentrations in periphyton communities after 2 h (panel A) and 7-days (panel B) of exposure to Cu and P treatments in laboratory streams. Error bars are standard deviation of two samples from the same stream. Low (50 μg/L) and high (250 μg/L) P media are represented by white and black bars, respectively. Control, low and high Cu treatments correspond to nominal levels of 0, 5 and 25 μg/L dissolved Cu, respectively. ND: not detectable.

Table 1
Chlorophyll *a* (Chl*a*), ash-free dry mass (AFDM) and autotrophic index (AI) of periphyton communities after seven-day exposure to different dissolved Cu and P conditions.

Cu media	P media	Chl*a* (μg/cm^2)	**Chl*a* change (%)**	AFDM (mg/cm^2)	**AFDM change (%)**	AI[a]	**AI change (%)**
Control	Low	10.9	**−35.9**	1.20	**−11.8**	109.8	**37.9**
Control	High	11.7	**−31.2**	1.21	**−11.1**	103.1	**29.5**
Low	Low	10.7	**−37.1**	1.17	**−13.6**	109.6	**37.6**
Low	High	13.1	**−22.9**	1.33	**−1.8**	101.7	**27.7**
High	Low	8.3	**−51.2**	1.31	**−3.6**	157.6	**97.9**
High	High	11.2	**−34.1**	1.37	**1.1**	122.4	**53.7**

Changes of Chl*a*, AFDM, and AI through time were calculated as a percentage of the initial value. Control, low and high Cu treatments correspond to nominal 0, 5 and 25 μg/L Cu, respectively. Low P and high P treatments represent nominal 50 and 250 μg/L, respectively.

[a] AI=AFDM/Chl *a*. Higher positive values indicate more changes towards heterotrophic dominance in periphyton community.

growth, peri+water exposure demonstrated a significant interaction between Cu and P treatments ($F_{2,24}=6.26$; $p<0.01$). Organisms exposed to no Cu grew at a faster rate than those exposed to the high Cu treatment irrespective of P treatment, whereas *H. azteca* exposed to the low Cu treatment grew faster under low P conditions and slower under high P (Fig. 3A). *H. azteca* in high Cu peri+water treatment grew at a rate comparable to those exposed to stream water without any food (Fig. 3A and B). For water treatments, organisms grew poorly in controls and better in water containing residual Cu (< 1.7 μg Cu/L) not absorbed by the biofilms (Fig. 3B).

The feeding rates of *H. azteca* in peri+water treatments showed that interestingly, the highest feeding rate occurred in high Cu treatments (Fig. 4), which indicates *H. azteca* unlikely has protective feeding behavior of avoiding Cu contaminated food. In addition, under both control and low, there were little differences in observed feeding rates between two P treatments (Fig. 4).

4. Discussion

4.1. Cu exposure to periphyton communities

Copper exposure is known to cause structural and functional changes in periphyton communities (Soldo and Behra, 2000; Barranguet et al., 2002; Guasch et al., 2002). In this study, experiment-wide biomass reduction (measured as chl*a*) and AI increase were observed in streams spiked with Cu as well as the control during experiment. Therefore, besides Cu and P, other environmental factors (e.g. light intensity, light spectrum, water temperature) that differed between the field and laboratory probably have also contributed to the decrease of biomass. Compared with control, effects of Cu on periphyton community structure were implied by AI shifts to a more heterotrophic community at high Cu level. P might have a counter effect with Cu by promoting the growth of autotrophic community, which is in accordance with previous studies suggesting phosphorus may increase copper tolerance of periphyton (Guasch et al., 2004; Serra et al., 2010).

4.2. The effects of Cu exposure on H. azteca

There is growing evidence that dietborne metal toxicity may be important in aquatic ecosystems (Borgmann et al., 2005; Clearwater et al., 2002; De Schamphelaere et al., 2004). In this study, under high Cu level (nominal: 25 μg/L), both high Cu concentration and high ingestion rate occurred, leading to high exposure levels which is probably associated with significant *H. azteca* growth reduction detected in both low and high P media. According to the energy allocation theory, the inhibition of growth can be explained by increased energy consumption and/or reduced energy acquisition (Kooijman, 2000; Nogueira et al., 2004). In this study, *H. azteca* seem to have highest feeding rates in high Cu treatments, so lowest growth rates in these treatments not likely result from reduced energy acquisition but energy consumption. One possible mechanism can be that organisms,

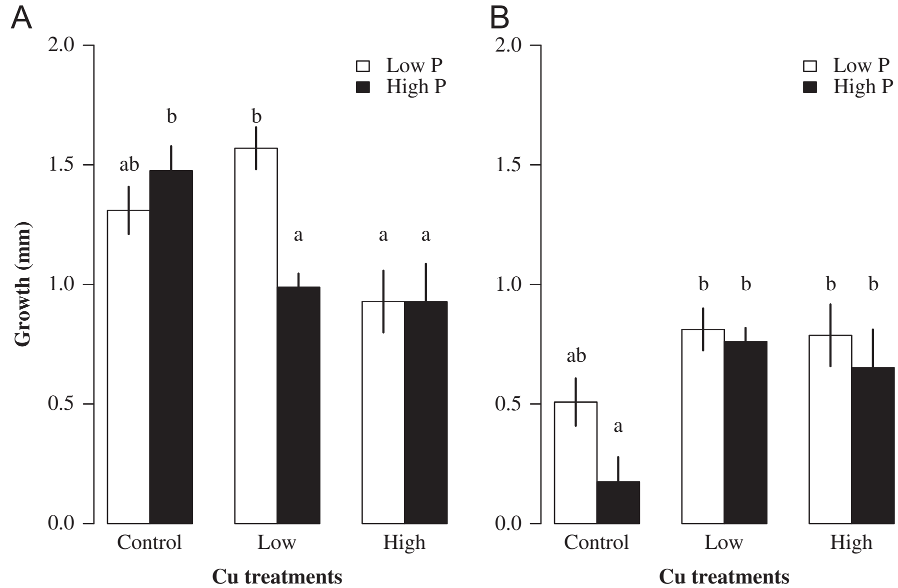

Fig. 3. Mean and standard error ($n=5$) of *H. azteca* growth in low P (white bars) and high P (black bars) treatments after 7-day combined (peri+water) exposure (panel A) and water exposure (panel B). Control, low and high treatments correspond to nominal 0, 5 and 25 μg/L dissolved Cu, respectively. Different letters indicate statistically significant differences among treatments in different exposures.

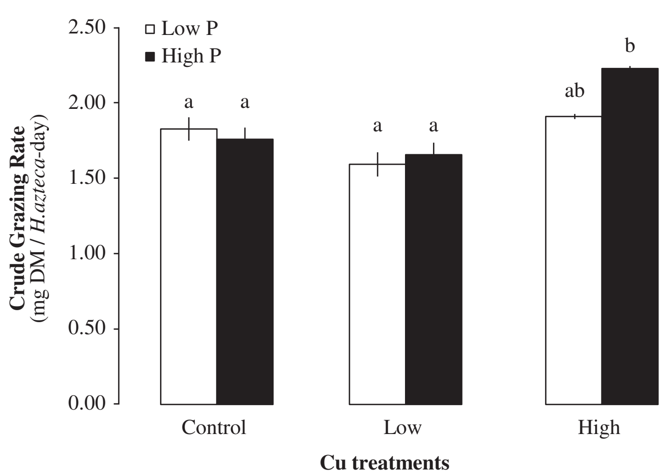

Fig. 4. Mean and standard error ($n=5$) of *H. azteca* crude feeding rates in low P (white bars) and high P (black bars) treatments during 7-day combined (peri+-water) exposure. Crude feeding rates were calculated by equation—[pre-grazing DM—post grazing DM]/7 days/10 *H. azteca*. Control, low and high treatments correspond to nominal 0, 5 and 25 μg/L dissolved Cu, respectively. Different letters indicate statistically significant differences among treatments in different exposures. DM: dry mass.

under high Cu exposure level increased metabolic costs to withstand toxicant stress like restoration of bio-molecules damaged by redox-cycling induced by accumulated Cu (Mason and Jenkins, 1995) or for detoxification processes such as metallothionein production or copper storage in granules (Bryan and Gibbs, 1983), compared to energy cost for those organisms in low Cu treatments and controls.

4.3. Discrepancy of H. azteca growth related to P levels at low Cu level

Depending on the exposure level, both positive and negative effects of dietary Cu on growth of aquatic invertebrates have been observed in previous studies, which highlight dual role of Cu as an essential element and toxicant (De Schamphelaere et al., 2004; De Schamphelaere et al., 2007). These contrasting effects of dietborne Cu exposure on invertebrates can be possibly attributed to hormesis-type concentration–response relation between Cu and digestive enzyme activity in benthic invertebrates (Chen et al., 2002). However, it is interesting to note that in this study, P seems to take the role of determining whether enhancement or inhibition of *H. azteca* growth occurs at the same low Cu exposure level.

Dietary toxicity studies for combined metals and phosphorus are scarce and the physiological processes governing metal bioaccumulation from diet are not fully understood (Croteau and Luoma, 2008). The idea currently well accepted is that uptake of metal from diet is a function of how much food an organism ingests, the metal concentration in the food and how much of that metal is extracted and assimilated into the feeding organism (Luoma and Rainbow, 2008). In this study, biomass-specific Cu concentrations in periphyton in high P treatments are slightly lower than in low P treatments. Therefore, no evidence supports that metal concentration in food is the reason of severe growth inhibition of *H. azteca* in the high P treatment. Furthermore, differences in growth of *H. azteca* seem also unlikely attributable to differences in *H. azteca* feeding rates between two P treatments at low Cu level. Although amphipod may prefer green algae or/and high P content algae stimulated by nutrients addition (Kraufvelin et al., 2006), in this study, no difference was found in crude feeding rates between low and high P treatments under low Cu condition (Fig. 4). While we believe crude feeding rates are important and meaning information for this study, we acknowledge these feeding rates can only be considered as crude estimation due to several underlying assumptions such as enough edible periphyton for grazing throughout the exposure period, no loss of dry mass due to microbial decomposition, and no tile to tile variation in pre-grazing dry mass of periphyton from the same stream; thus, some formal and comprehensive feeding bioassays would probably provide more convincing results.

At last, since both feeding rates of organism and metal concentration in the food cannot explain the reduced growth, to best of our knowledge based on this study, higher assimilation efficiency of Cu in high P treatment then becomes the most plausible mechanism. Previous studies have shown that physiological condition (different growth phases) of algal food could affect assimilation efficiencies in invertebrates (Barofsky et al., 2010; Reinfelder and Fisher, 1991). In this study, the temporal changes in chl*a* over the one-week exposure implies that algal biomass declined the least in the low Cu-high P treatment, which might suggest algae exposed to this combination stay in a different growth phase from other treatments, such as the end of stationary phase for low Cu- high P treatment and death phase for others. Another mechanism possibly affecting assimilation would be changes in distribution or/and speciation of the Cu stored in the periphyton community. It is known that trace metals partition within algal cells (Luoma et al., 2008) and can be stored in granules or bound to phytochelatins (Mason and Jenkins, 1995), but there is little evidence that any speciation represented the sole form of metal that is bioavailable for trophic transfer to a herbivore (Luoma et al., 2008). Under P-repleted conditions, algal cells can undergo luxury uptake of P, often storing the excess P as polyphosphate bodies (PPB), which are proposed to have a high affinity for divalent metals like Cu (Serra, 2009; Tlili et al., 2010). This research seems to suggest that Cu-polyphosphate complex in periphyton might be important for linking aqueous nutrient condition to dietary metal exposure to *H. azteca*. Although this mechanism is one possible explanation for the observed results, the implications of PPB for metal uptake and food-chain transfer efficiency are not known and clearly require more study. Indeed, intracellular speciation of Cu in periphyton and its bioavailability for grazers deserve further research efforts.

If Cu assimilation rates do differ between low and high P conditions, it is interesting to note that we did not measure any difference in growth between our P treatments under high Cu conditions. Here, we believe that even with low Cu assimilation under low P, the high Cu concentrations make the dietary dose great enough to lead to severe reduced growth, similar to the growth rates of amphipods given no food. In brief, we suggest that assimilation efficiency affected by P levels may only be important for distinguishing *H. azteca*'s physiological outcomes due to dietary exposure under lower Cu conditions, thus explaining the observed interactive effect of Cu and P.

Besides possible effects due to dietary toxicity of Cu, shifts in periphyton community and correspondingly, its nutritional quality may also contribute to the discrepancy of growth rates in two P levels. It has been well described that both copper and phosphorus exposure can cause a large variety of structural and functional changes in periphyton communities (Barranguet et al., 2002; Guasch et al., 2002; Soldo and Behra, 2000; Tlili et al., 2010; Vermaat, 2005). The difference in composition of periphyton communities in streams may result in distinct diet compositions thus different food quality of *H. azteca* among treatments. Unfortunately, though our AI data indicated various extent of community shifts towards heterotrophic, the exact differences in community structure and nutritional quality of periphyton were not examined. Hence, this postulated mechanism could not be further verified by this study and merit future efforts.

5. Conclusions and research perspectives

Cu has been commonly used as algaecide for preventing and treating algal bloom caused by eutrophication in aquatic ecosystem (Chorus and Bartram, 1999), however, the risk of concurrent Cu pollution and eutrophication for higher trophic-level organisms is unclear. This study attempts to fill in the gap by directly exploring the risk of waterborne Cu under different P conditions to grazers via periphyton in laboratory. Results suggest potential synergistic effect of Cu and P in water on the growth of herbivores at low Cu level. Though no single explanation for this synergism is definitive, several postulated mechanisms related to differences in *H. azteca* assimilation efficiency of metal due to algal physiological conditions, changes in biofilm internal metal partitioning, and differences in nutritional quality may provide some useful insights for future investigations. To determine if eutrophication can considerably enhance the potency of metal pollution on ecosystems, corresponding field studies on freshwater invertebrates at the population or community level is needed.

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