Interactive effects of phosphorus and copper on *Hyalella azteca* and periphyton

by

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Abstract

Eutrophication is known to be frequently associated with metal pollution in aquatic ecosystems. This research examined the interaction between dissolved copper and phosphorus, with respect to their effects on the growth of a freshwater amphipod *Hyalella azteca* feeding on periphyton. The study design included two tiers: (1) a laboratory stream experiment where natural periphyton communities accumulated Cu under a gradient of Cu and P concentrations; and (2) a beaker experiment where *H.azteca* were exposed to water and periphyton from laboratory streams. There was rapid Cu accumulation by periphyton but the total Cu concentration of periphyton was not directly related to the dissolved P treatment in the stream experiment. In terms of *Hyalella* growth, an interactive effect was found between Cu and P as high phosphorus concentration was related to reduced growth at relatively lower Cu concentration. Our findings suggest that eutrophication may result in greater Cu toxicity to benthic macroinvertebrates as a result of dietary exposure from periphyton.

Keywords: Cu toxicity; Phosphate; Interaction; Dietary exposure

1   Introduction

Aquatic environments can be highly affected by metal pollution, as they are an ultimate receptor of urban wastewater, industrial and mine effluents, agriculture runoff and atmospheric deposition (Nriagu, 1979). However, aquatic ecosystems are rarely disturbed by a single type of stressor (Ivorra, et al., 2002), and the impact of pollutants may interact with various abiotic and biotic stressors (Heugens, et al., 2001). Consideration of multiple-stressor scenarios has been recognized to be a critical component in the derivation of risk assessments that simulate real world conditions (Hope, 2006). In aquatic ecosystems, eutrophic conditions are found frequently associated with metal pollution (Lopez-Flores, et al., 2003), though both problems have been extensively investigated but traditionally treated as separate. To better understand the impact of metal toxicants 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. One approach becoming common 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 grow at the interface between the overlaying water and the sediments (Hill, et al., 2010), thus providing an integrated representation of the accumulation of toxicants in the aquatic environment (Newman and McIntosh, 1989; Lowe and Pan, 1996). Because of their sorptive nature and large surface area (Hill, et al., 2010), periphyton can take up heavy metal from the water, producing an internal

concentration often thousands of times greater than that of their surrounding water

(Genter, 1996; Hill, et al., 1996).

Metal toxicity and accumulation in algae or periphyton have been broadly studied in the

past (Sabater, et al., 2007). Studies have shown that the uptake of dissolved metal could

lead to decreased periphyton biomass and altered taxonomic composition (Guasch, et al.,

2002; Roussel, et al., 2007; Serra and Guasch, 2009). Recent studies of the interactive

effect of trace metals and nutrients on periphyton have found higher metal tolerance in

periphyton communities grown under more eutrophic conditions (Serra, et al., 2010).

This greater metal tolerance may lead to less reduction in periphyton biomass but higher

metal accumulation in periphyton, which could be potentially toxic to herbivores that

consume them. Despite the implications of these recent investigations, little information

is available on how greater metal tolerance of periphyton in the presence of nutrients

affects higher trophic-level organisms like benthic macroinvertebrates or fish.

The purpose of this research was to evaluate the influence of nutrients on periphyton-

mediated metal toxicity to stream macroinvertebrates. We specifically focus on copper

(Cu) and phosphorus (P) which are commonly found together in fluvial systems draining

industrial and urban watersheds (Twiss and Nalewajko, 1992). It is hypothesized that

periphyton grown under eutrophic conditions will contain relatively more Cu, which in

turn poses a greater risk of dietary Cu exposure to consumers of periphyton. To test this

hypothesis, experiments were conducted 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   This study was conducted in two tiers as shown in Fig.1: (1) A laboratory stream experiment in which field-collected periphyton communities were exposed to different nutrient and metal conditions in indoor recirculating streams; and (2) a beaker experiment where *H.azteca* were exposed to water and periphyton from the mesocosms. Each of six streams included one combination of dissolved Cu and P concentrations to simulate Cu exposure of periphyton in oligotrophic or eutrophic conditions.

2.1   Laboratory stream experiment

54 Unglazed tiles (26 cm^2) were placed in the raceway of Saline Fisheries Research Station, Saline, MI from August to October, 2010 to allow colonization of a natural periphyton community. After colonization, the tiles were transported to the University of Michigan Aquatics Laboratory (School of Natural Resources and Environment) and randomly distributed 9 tiles to one of 6 streams. Test water, approximately pH 7.9, temperature 24°C, dissolved oxygen10mg/L, alkalinity 80mg/L, hardness 100mg/L, and conductivity 344μs/cm on day 0, was continuously recirculated in streams during exposure period.

Each of six streams included single combinations of dissolved Cu (3 treatments) and P (2 treatments) in reconstituted water (EPA, 2000) to simulate Cu exposure of periphyton in oligotrophic or eutrophic conditions. For Cu exposure, three concentrations were studied: control (0 ug/L), the low Cu (5 µg/L), and the high Cu (25 µg/L). For P, two concentrations 50 PO_4-P µg/L and 250 PO_4-P µg/L were used to mimic relatively oligotrophic and eutrophic conditions in aquatic ecosystems. During the week-long exposure, water was continuously recirculated in the streams. On days 0 and 3, Cu and phosphate was added to maintain concentrations at treatment levels. During the experiment, illumination was provided by fluorescent grow lights (approximately 2000 lux, Hydrofarm) with a 16:8 hr 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 was scraped from tiles, homogenized, and collected onto a preweighed glass fiber filters (pore size 0.7µm). 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).

2.2   Beaker experiment for 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 day old laboratory-cultured *H.azteca* (EPA, 2000). Two types of treatments for each stream, each replicated 5 times, were designed to separate waterborne exposure and dietary exposure. Periphyton-excluded (PE) treatments exposed *H.azteca* to stream water and one uncolonized tile while the periphyton-included (PI) treatments exposed *H.azteca* to combined periphyton and stream water. There are 10 *H.azteca* in each replicate. After one week, the organisms were collected to measure survival, growth, and tissue Cu content and periphyton was sampled as above for biomass and Cu content. 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 body length (±0.05 mm) (EPA, 2000) and body Cu content. Individual *H. azteca* were photographed at a magnification of 5X and measured using ImageJ 1.43u (National Institutes of Health).

Similar to methods used in Serra et al. (2009), 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). The digestion of *H.azteca* tissue samples (non-depurated)

was accomplished using the methods described by Norwood et al. (2006). Digested tissue and periphyton were then analyzed by ICP-OES (detection limit of Cu—1µg/L).

2.3 Data analysis

Ratios of AFDM to chlorophyll *a* of periphyton, called autotrophic index (AI: high AI represents low autotrophy level), is calculated to investigate the effects of phosphorus on periphyton composition. Comparisons of Cu content and biomass of periphyton as well as *H. azteca* growth among Cu and P treatments were made using two-way ANOVA tests and Tukey's honest significance test. All statistical analyses were performed using R version 2.12.1 (R Development Core Team 2010) and in all cases, an alpha level 0.05 was chosen to interpret the significance of the effects and differences among treatments, which indicated by alphabetical letters in figures. No violation for normality and equal variance were found. No statistical analysis was taken for Cu content of *H. azteca* due to the single sample for each treatment.

3 Results

3.1 Laboratory stream experiment

Most of physical and chemical parameters of stream water did not show great differences among the six streams (Table A1). The stream water was circum-neutral with pH = 8 and alkalinity around 80 mg/L $CaCO_3$. A gradual increase in conductivity and hardness was observed during one week (Table A2), likely due to water evaporation and daily addition of reconstituted water. Periphyton rapidly adsorbed Cu from streamwater as dissolved Cu

concentration three days after spiking decreased to less than 1.8µg/L. Rapid absorption of Cu can also be seen in the elevated (though variable) Cu concentrations within periphyton just 2 hrs post exposure but no significant difference among treatments (p>0.05) (Fig.2A). After 7-day exposure, periphyton Cu concentrations were significantly greater in the high Cu treatment compared to low Cu and reference treatments, with slightly lower Cu in high P treatments (Fig.2B).

Both chl *a* and AFDM were higher in the high P treatment than in the low P treatment (Fig.3), which suggested greater food availability in high P treatments. Periphyton exposed to the high Cu treatment showed decreased chl *a* but an increase in AFDM relative to the controls. Periphyton in the low Cu treatment exhibited a slight growth enhancement rather than an adverse effect on biomass. It is worth noting that periphyton in low Cu and high P treatment presented an increase in both chl *a* (3.5%) and AFDM (14.6%) after one-week exposure, however, all the other treatments have decreased chl *a* and various AFDM responses (Table 1). For autotrophic periphyton, all treatments showed an increase in AI (AFDM/Chl *a*) during the experiment, which indicates that algae did not grow as well in the lab as in the field. Elevated phosphorus seems to have a strong effect on enhancing autotrophic algae growth (lower AI increase) and/or compensate for Cu toxicity on algae at high Cu level. The biofilm community under high Cu exposure, shifted to dominance by heterotrophs in low P treatment (AI difference=152.40%) but there was only a slight shift in AI, similar to it is in control groups, in high P treatments (Table 2).

3.2   Beaker experiment for 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 PI or PE exposures ($p > 0.05$). For *H. azteca* growth, PI exposure demonstrated a significant interaction between Cu and P treatments (Fig.4A, $F_{2,24} = 6.26$; $p < 0.01$). Organisms exposed to no Cu grew at a faster rate that 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 ($p <0.0001$)(Fig. 3A). *H. azteca* exposed to high Cu and low Cu in combination with high P grew at a rate comparable to *H. azteca* exposed to just streamwater without any food (Fig.4A-B). Organisms grew better in water containing 0-1.7 μg Cu/L than in control PE treatments (Fig. 3B). This could result from effect of Cu as essential micronutrients for the enzymes and haemocyanin but may also attribute to higher hardness (Table A1) in control groups.

The average Cu concentration of *H.azteca* exposed to periphyton and water (67.2 μg Cu/g DW) was almost twice the levels of those exposed to water only (37.5 μg Cu/g DW) (Fig.5), which implies that dietary exposure to periphyton contributed a considerable portion of Cu to tissue. Within PI exposures, *H. azteca* in the control Cu treatment groups had on average 37.52 μg Cu/g DW which is close to the estimated theoretical total body Cu metabolic requirement of 38.1μg/g DW.

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), specifically by reducing algal abundance (Roussel, et al., 2007; Serra and Guasch, 2009). Hence, it is not surprising to observe algal biomass reduction (measured as chl *a*) in streams spiked with Cu during experimental exposure. However, since periphyton communities grown in the control treatments also showed a decrease in algal biomass (Table 1), other environmental factors (e.g. light intensity, water temperature) that differed between the field and laboratory may have contributed to the experiment-wide reduction in periphyton biomass.

Compared with control, little adverse effects on biomass of periphyton community were observed and this indicates that the nominal aqueous exposure Cu levels 5 and 25 μg/L to periphyton may not be sufficient to cause dramatic change in algal biomass within one week. Similar responses were found in recent copper study in a eutrophic river that no observed effect at periphyton community level at 5 or 4 μg /L and lowest observed effect at 25 or 20 μg/L (Roussel, et al., 2007).

Previous studies already demonstrated the variation in responses of algal communities to Cu under a variety of environmental factors like light, nutrients, water pH and alkalinity (i.e.Guasch, et al., 2002; Navarro, et al., 2002). Results of this study showed phosphate

reduced adverse effects on periphyton biomass and autotrophic index shift at all Cu levels including controls, which implies phosphorus may increase copper tolerance of periphyton (Guasch, et al., 2004; Serra, et al., 2010). One of the underlying mechanisms generally accepted for this is related to intracellular polyphosphate bodies (PPB) which can sequester metals in a detoxified form (Baxter and Jensen, 1980; Twiss and Nalewajko, 1992).

4.2   The effects of Cu exposure on *H.azteca*

There is growing evidence that dietborne metal toxicity may be important in aquatic ecosystems (Clearwater, et al., 2002; De Schamphelaere, et al., 2004; Borgmann, et al., 2005). In this study, significant growth reduction was detected under high Cu treatments (nominal: 25μg/L) for both low and high P media. According to the energy allocation theory, the inhibition of growth may be explained by increased energy consumption and/or reduced energy acquisition (Kooijman, 2000; Nogueira, et al., 2004). On one hand, increased metabolic costs may be required to withstand toxicant stress like restoration of bio-molecules that are damaged by redox-cycling induced by accumulated Cu (Mason and Jenkins, 1995) or for detoxification processes such as metallothionein production (Amiard, et al., 2006) or copper storage in granules (Bryan and Gibbs, 1983). On the other hand, inhibition of food ingestion (Allen, et al., 1995) or/and reduced food assimilation efficiency as a result of inhibition of digestive enzyme activity (Chen, et al., 2002) can contribute to reduced energy acquisition. Furthermore, metal contaminated food may have had a reduced nutritional quality and growth reduction is an indirect result

of food quality rather than a direct toxicological impact of ingested metal (De Schamphelaere, et al., 2007). Though distinct growth inhibition was observed between low and high Cu treatments, similar Cu body burden was found in *H.azteca* tissue. This finding supports the idea that *H.azteca* may be able to regulate (i.e., excrete) copper and body burdens are not a useful indicator of exposure or potential toxic effects (Borgmann, et al., 1993; Langston and Spence, 1995). One of the possible explanation for this is that when the quantity of metal-containing granules held in the cytoplasm of sequestering tissues reaches certain limits, they are excreted (Taylor and Anstiss, 1999). In this study, it is likely that Cu exposure levels are within the range of *H.azteca*'s capability to regulate, but higher energy cost on Cu excretion under higher Cu treatments result in lower growth in these treatments. However, for testing tissue concentration of organisms, gut clearance is recommended to carry out before acid digesting organisms, but unfortunately, this step was not included in this experiment, thus the tissue concentrations may be overestimated due to Cu-contaminated periphyton in gut.

4.3   Discrepancy of growth inhibition of *H.azteca* related to P levels

Both positive and negative effects of dietary Cu on growth of aquatic invertebrates have been observed, which highlights its dual role as an essential element and toxicant (De Schamphelaere and Janssen 2004, De Schlamphelaere et al. 2007b). These contrasting effects of dietborne Cu exposure on invertebrates can be probably attributed to hormesis-type concentration–response relation between Cu and digestive enzyme activity in benthic invertebrates (Chen, et al., 2002). Interestingly, at the same low Cu exposure

level both slight enhancement and significant inhibition were observed on *H.azteca* growth with P exposure differentiating between the two responses. Despite 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), some information about toxicity for dietborne metals may contribute to understanding the mechanism of this joint effect of Cu and P. 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 low P treatments are slightly higher than it is in high P treatments. Therefore, the metal concentration is probably not the reason for growth inhibition of *H.azteca* in the high P treatment and the observed differences in growth are most likely due to alterations in ingestion and assimilation.

According to the temporal changes in chl *a* over one-week exposure, algal biomass declined in all treatments but the high P and low Cu treatment, which perhaps suggest algae exposed to this combination stay in a different growth phase from other treatments, possibly stationary phase for low Cu, high P treatment and death phase for others. As physiological condition (different growth phases) of the phytoplankton food could affect both uptake preference and assimilation efficiencies of metals in invertebrates (Reinfelder and Fisher, 1991; Barofsky, et al., 2010), it might be possible that rigorous

growth inhibition of *H.azteca* in high P treatments results from higher ingestion rate of Cu-contaminated food or/and assimilation efficiency of Cu in this treatment.

Furthermore, the difference in community composition may also account for potential distinct feeding rate of *H.azteca*. It has been well described that copper exposure can cause a large variety of structural and functional changes in periphyton communities (Soldo and Behra, 2000; Barranguet, et al., 2002; Guasch, et al., 2002) and the shift in AI detected in this study also implied this point. Meanwhile, enhanced nutrient availability can also lead to shifts in taxonomic composition (Vermaat, 2005). Tlili, et al (2010) recently demonstrated that relative percentages of algae species in periphyton were influenced by both exposure to Cu and P gradients, indicating that increases in P lead to a higher proportion of green algae and less diatoms. It is worth mentioning that the percent cover of green algae stimulated by nutrients addition was found significantly related to the abundance of amphipods (Kraufvelin, et al., 2006), thus, green algae or/and high P content algae may be amphipod's preferred food within periphyton. *H.azteca,* which may prefer green algae, could have consumed more periphyton in high P treatments and therefore increase the ingestion rate of biofilm-bound Cu. In controls, similar discrepancy was not observed probably due to sufficient food availability in either P treatment without Cu stress. Moreover, since periphyton accumulated very high concentration of Cu under high Cu exposure (more than 10 folders higher than it was at low Cu level), ingestion rate, even it differs between high and low P treatments, may not be the determinative factor for the dose of Cu in the high Cu exposure.

Another mechanism explaining the Cu-P interaction on *H.azteca* growth would be

changes in distribution or/and speciation of the Cu stored in the periphyton community.

Within algae, metals can be stored in granules or bound to phytochelatins (Mason and

Jenkins, 1995), and this may alter the bioavailability of dietborne metals (Clearwater, et

al., 2002). Under P-repleted conditions, algal cells 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, Ahmed et al. 2010). Although this

mechanism is a possible explanation for observed results, the implications of PPB for

metal uptake and food-chain transfer are not known and require further study. It is

known that trace metals partition within algal cells (Luoma, et al., 2008) but there is little

evidence that any fractions represented the sole form of metal that is bioavailable for

trophic transfer to a herbivore (Luoma, et al., 2008). This research suggests that Cu-

polyphosphate complex in periphyton might be important for linking nutrient condition to

dietary metal exposure to *H.azteca*. Indeed, intracellular speciation of Cu in periphyton

and its bioavailability for grazers certainly deserve further research efforts.

As it is well known that periphyton communities are composed of living and dead algal,

bacterial cells, particulate detritus and inorganic particles bound within an organic matrix

of largely extracellularlly released polysaccharide secretions(Azim, 2005). These

extracellular polymeric substances (EPS) contain high amounts of negatively charged

functional groups such as carboxyl, phosphate, and sulphate groups and can potentially

act as metal-binding sites (Kaplan, et al., 1988; García-Meza, et al., 2005). Though studies showed that some algae exposed to Cu produced extracellular metal ligands (Moffett and Brand, 1996), the combined effect of Cu with P on inducing EPS secreted by algal and bacterial communities is not clear to date. More efforts are probably needed for explaining the role of heterotrophic periphyton communities in posing risk to their consumers through food chain.

Study limitations: this experiment is limited by the number of replicate streams and analytical replicates for testing Cu in periphyton and its grazers, but the trend of Cu accumulation in periphyton over time and the Cu effects on growth of periphyton and *H.azteca* were generally captured in this study. Lack of identification of periphyton community composition (e.g. algal species), feeding rate of *H.azteca* and distinguishing intracellular/extracelluar Cu make it difficult to verify potential mechanisms.

5   Conclusions and research perspectives:

In the context of assessing the impacts of pollution on ecosystem, one of the most critical points is to evaluate risk from multiple stressors and understand stressor interactions. To our knowledge, this is the first research which sought to directly link waterborne Cu and P exposure to metal accumulation in periphyton as well as the responses of periphyton grazers in laboratory. Results demonstrate the synergistic effect of Cu and P in water, in the respect of adverse effects to herbivores mostly through dietborne exposure. Our findings imply that eutrophication may considerably enhance the potency of metal

pollution to freshwater invertebrates, especially at metal concentrations below standard toxicity benchmarks. The mechanism is possibly related to nutrient-induced differences in algal physiological condition or metal partitioning within algal cells however undoubtedly deserves further research.

Tables and Graphs
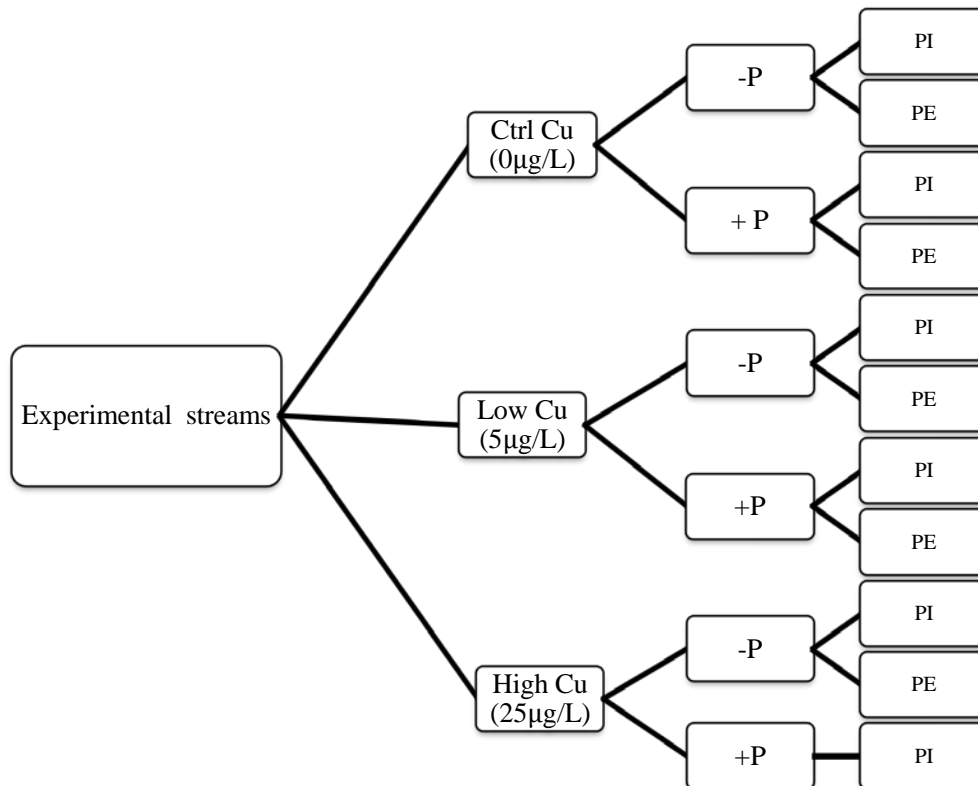
Fig. 1. Scheme of the experimental design used in the six experimental streams. Cu-media refers to the nominal copper concentrations in water during one-week laboratory stream experiment. P-media refers to the nominal phosphorus conditions in water. –P and +P represent nominal dissolved phosphorus concentrations 50 µg/L and 250 µg/L, respectively. Beaker experiments on *H.azteca* include two treatments (periphyton included and periphyton excluded) for each Cu-P combination in order to distinguish the toxicity of dietary exposure through periphyton. Each treatment has five replicates with 10 *H.azteca* in one replicate.
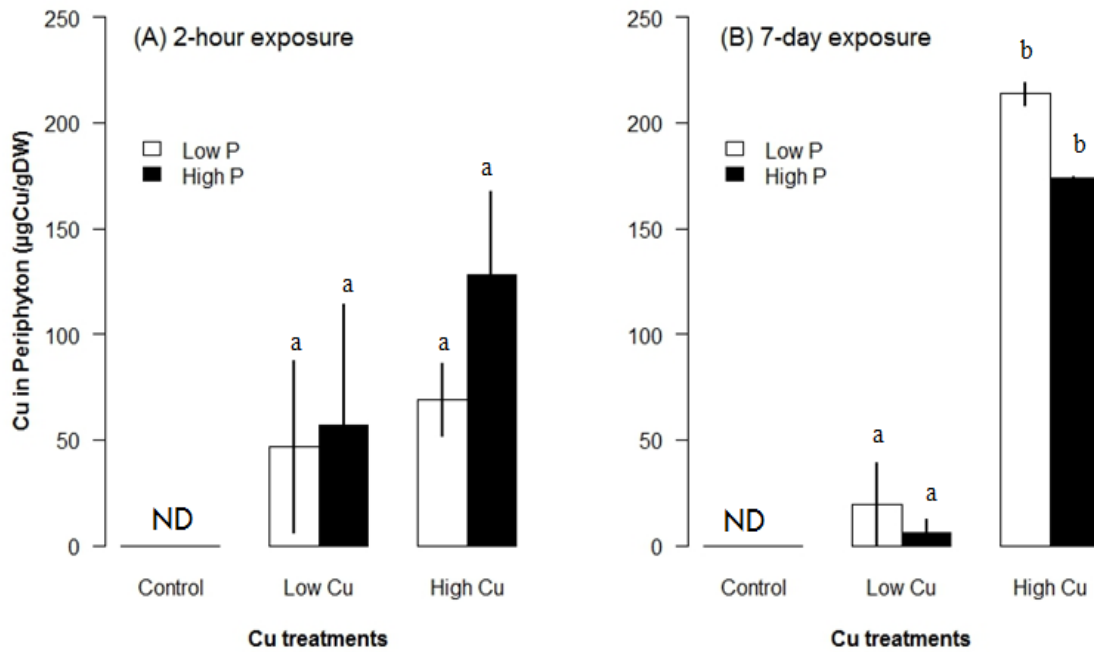
Fig.2. Mean and standard error (n = 2) of biomass-specific total Cu concentrations in periphyton communities after 2-hour (panel A) and 7-day (panel B) incubations in Cu and P treatments in an artificial laboratory stream. Low P media (50 µg/L) and high P media (250 µg/L) are represented by white and black bars, respectively. Control, low and high Cu treatments correspond to nominal levels of 0, 5 and 25 ug/L Cu in water, respectively. ND: not detectable. Alphabetical letters indicate statistical significance among treatments. (Data source: Table A9&A10)
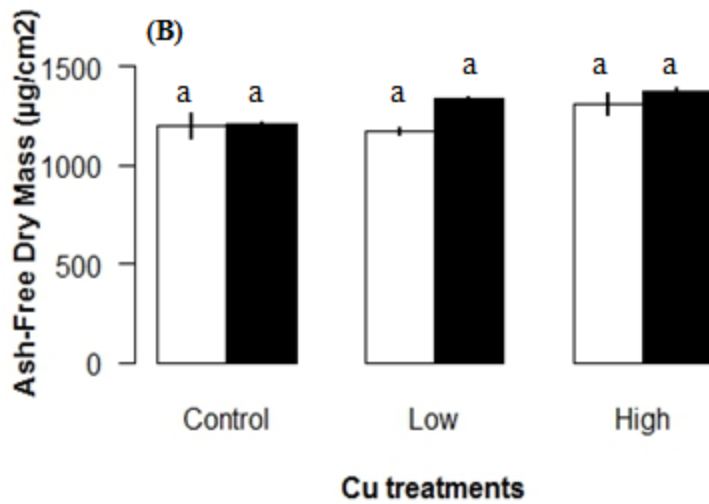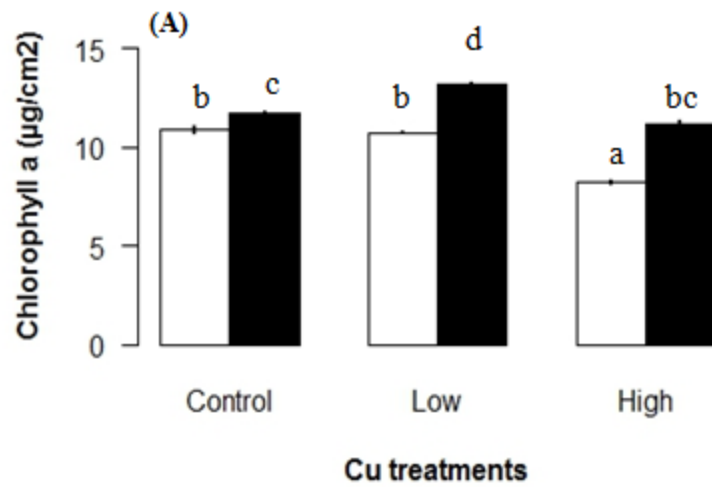
Fig.3. Mean and standard error (n = 2) of periphyton chlorophyll a content (panel A) and ash-free dry mass (panel B) in low P media (white bars) and high P media (black bars) in an artificial laboratory stream. Control, low and high Cu treatments correspond to nominal 0, 5 and 25 ug/L Cu in water, respectively. Alphabetical letters indicate statistical significance among treatments. (Data source: Table A6 & A8)
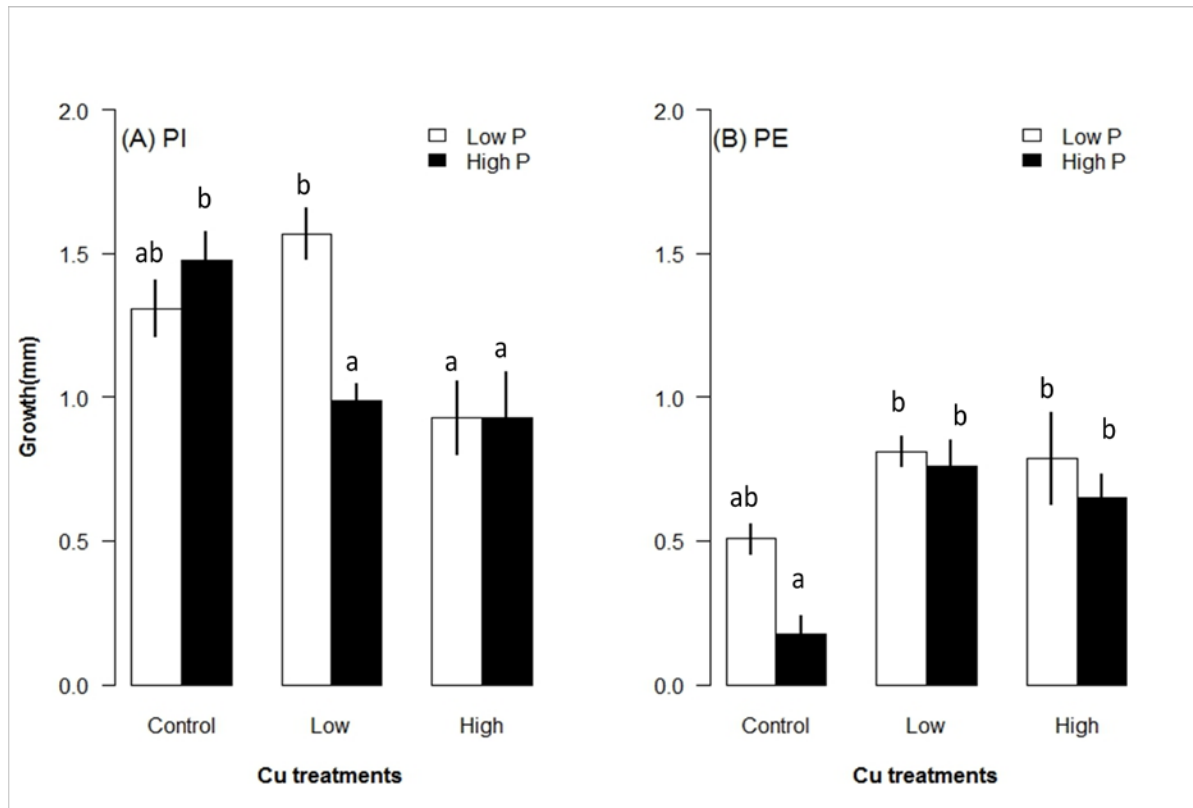
Fig.4. Mean and standard error (n = 5) of *H.azteca* growth in low P treatments (white bars) and high P treatments (black bars) after 7-day periphyton-included (PI) exposure (panel A) and periphyton-excluded (PE) exposure (panel B. Control, low and high treatments corresponds to nominal 0, 5 and 25 μg/L Cu in stream where periphyton previously grew, respectively. Alphabetical letters indicate statistical significance among treatments in PI and PE. (Data source: Table A12 & A13)
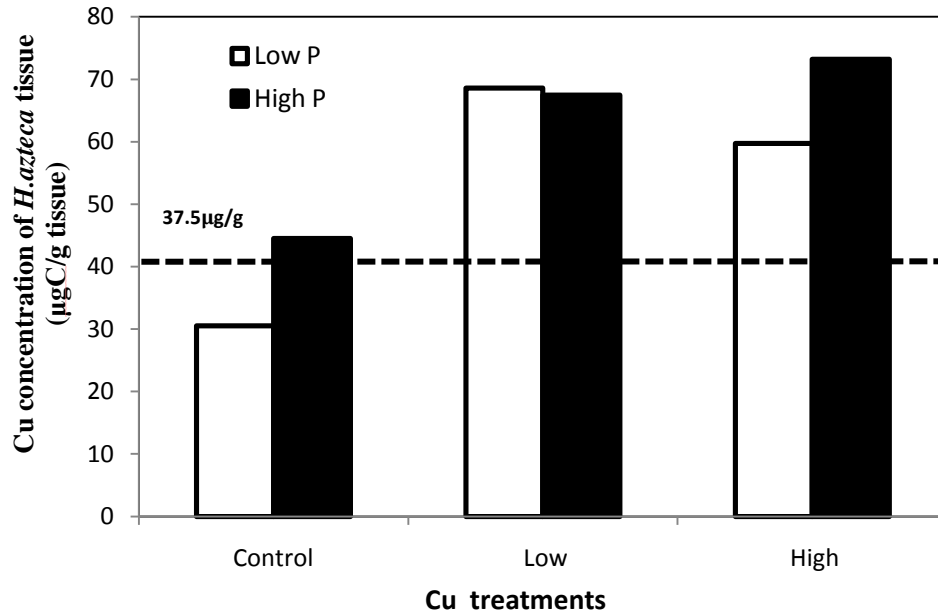
Fig.5. Cu concentrations in *H.azteca* tissue in low P treatments (white bars) and high P treatments (black bars) after 7-day combined periphyton and water exposure. Dash line indicates 37.5µg/g, the average Cu concentration of *H.azteca* exposed to water only exposure. Control, low and high Cu treatments corresponds to nominal 0, 5 and 25 µg/L Cu in stream where periphyton grew, respectively. (Data source: Table A11).

Table 1. Chlorophyll *a* (Chl *a*) and ash-free dry mass (AFDM) of periphyton communities under all treatments over one week in streams (Data source: Table A5-A8).

Cu media	P media	Chl *a* (day 1) ($\mu g/cm^2$)	Chl *a* (day 7) ($\mu g/cm^2$)	Chl *a* difference (%)	AFDM (day 1) ($\mu g/cm^2$)	AFDM (day 7) ($\mu g/cm^2$)	AFDM difference (%)
Control	Low	24.3	10.9	-55.3%	1841.1	1196.7	-35.0%
Control	High	18.1	11.7	-35.1%	1356.6	1206.4	-11.1%
Low	Low	16.2	10.7	-33.8%	1453.5	1172.5	-19.3%
Low	High	12.7	13.1	3.5%	1162.8	1332.4	14.6%
High	Low	17.0	8.3	-51.4%	1065.9	1308.1	22.7%
High	High	13.9	11.2	-19.6%	1259.7	1371.1	8.8%

Change sof Chl a and AFDM between day1 and day 7 were calculated and represented in percentage. Low and high Cu treatments correspond to nominal 0, 5 and 25 μg/L Cu in streams. Low P and high P treatments represent nominal 50 and 250 μg/L, respectively.

Table 2. Autotrophic index (AI) of periphyton communities under all treatments over one week in streams.

Cu media	P media	AI (day 1)	AI (day 7)	**AI difference (%)**
No Cu	Low P	75.70	110.07	**45.40**
No Cu	High P	75.08	102.86	**36.99**
Low Cu	Low P	89.90	109.52	**21.83**
Low Cu	High P	91.67	101.46	**10.68**
High Cu	Low P	62.84	158.60	**152.40**
High Cu	High P	90.60	122.60	**35.31**

AI difference between day1 and day 7 were calculated and represented in percentage. Low and high Cu treatments correspond to nominal 0, 5 and 25 μg/L Cu in streams. Low P and high P treatments represent nominal 50 and 250 μg/L, respectively. (Data source: Table A5-A8)

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Appendices

Table A1 Physical and chemical parameters measured for six experimental streams in one week. Low and High Cu media refer to the dissolved copper concentrations 5 µg/L and 25 µg/L; Low and High P media refer to the dissolved phosphorus conditions 50 µg/L and 250 µg/L in water, respectively.

Treatments		Parameter						
Cu media	P media	Light intensity (Lux)	pH	Temp (ºC)	Conductivity (µS/cm)	DO (mg/L)	Alkalinity (mg/L)	Hardness (mg/L)
N/A	Low	2480 (85)	8.03 (0.08)	25.7 (1.0)	413(29)	9.65(0.25)	88(0.0)	124(8)
N/A	High	2040 (103)	8.02 (0.07)	25.6 (1.0)	411(30)	9.63(0.25)	83(1.0)	124(16)
Low	Low	2100 (76)	7.85 (0.09)	26.2 (1.1)	364(24)	9.55(0.23)	72(22.0)	107(13)
Low	High	2377 (148)	7.91 (0.09)	26.1 (1.2)	367(41)	9.59(0.26)	84(4.0)	105(15)
High	Low	2337 (58)	7.97 (0.09)	26.0 (1.2)	399(36)	9.63(0.27)	78(6.0)	108(16)
High	High	2557 (171)	8.00 (0.09)	25.7 (1.1)	404(27)	9.69(0.27)	84(0.0)	116(12)

The values are the average and the standard error (in parenthesis) for each treatment.

Table A2 Data of alkalinity and hardness before and after 7- day stream experiment. Low and High Cu media refer to the dissolved copper concentrations 5 µg/L and 25 µg/L; Low and High P media refer to the dissolved phosphorus conditions 50 µg/L and 250 µg/L in water, respectively.

Treatments		Alkalinity (mg/L)		Hardness (mg/L)	
Cu media	P media	pre-test	post-test	pre-test	post-test
N/A	Low	88	88	116	132
N/A	High	82	84	108	140
Low	Low	94	50	94	120
Low	High	88	80	90	120
High	Low	72	84	92	124
High	High	84	84	104	128

Table A3 R output of ANOVA table for testing effects of Cu and P on periphyton-included *H.azteca* treatments

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
P	1	0.14477	0.14477	2.3618	0.1374223
Cu	2	1.17313	0.58656	9.5692	0.0008793
***					
P:Cu	2	0.76770	0.38385	6.2621	0.0064798
**					
Residuals	24	1.47113	0.6130		

- Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
- Shapiro-Wilk normality test: W = 0.9784, p-value = 0.7807

Table A4 Results from Tukey multiple comparisons of means of different periphyton-included *H.azteca* treatments

P:Cu media	diff	lwr	upr	p adj
250:0-50:0	0.1656	-0.3185492	0.649749213	0.8930917
50:5-50:0	0.2600	-0.2241492	0.744149213	0.5691065
250:5-50:0	-0.3210	-0.8051492	0.163149213	0.3453269
50:25-50:0	-0.3810	-0.8651492	0.103149213	0.1846879
250:25-50:0	-0.3824	-0.8665492	0.101749213	0.1817628
50:5-250:0	0.0944	-0.3897492	0.578549213	0.9898079
250:5-250:0	-0.4866	-0.9707492	-0.002450787	0.0483409
50:25-250:0	-0.5466	-1.0307492	-0.062450787	0.0205627
250:25-250:0	-0.5480	-1.0321492	-0.063850787	0.0201450
250:5-50:5	-0.5810	-1.0651492	-0.096850787	0.0123425
50:25-50:5	-0.6410	-1.1251492	-0.156850787	0.0049431
250:25-50:5	-0.6424	-1.1265492	-0.158250787	0.0048373
50:25-250:5	-0.0600	-0.5441492	0.424149213	0.9987833
250:25-250:5	-0.0614	-0.5455492	0.422749213	0.9986408
250:25-50:25	-0.0014	-0.4855492	0.482749213	1.0000000

Note: stream treatments explanation for following tables.

Treatment #	Cu media	P media
1	Low	Low
2	Low	High
3	High	Low
4	High	High
5	N/A	Low
6	N/A	High

Table A5 Data of Chlorophyll *a* of periphyton community at the start of 7-day stream experiment

Stream treatments	Volume of aliquots (ml)	Total volume (ml)	Chl *a* (ug/L)	Chl *a* (ug/cm^2)
1-A	5	500	329.5	12.77
1-B	5	500	504.8	19.57
2-A	5	500	331.3	12.84
2-B	5	500	323.2	12.53
3-A	5	500	442.9	17.17
3-B	5	500	432.4	16.76
4-A	5	500	301.5	11.69
4-B	5	500	415.9	16.12
5-A	5	500	624.6	24.21
5-B	5	500	630.3	24.43
6-A	5	500	457.2	17.72
6-B	5	500	475.1	18.41

Note: There are two replicates (A, B) for each stream treatment. Periphyton from 2 tiles were scraped by a hard-bristled toothbrush and diluted to 500ml, then 5ml aliquot of periphyton slurry filtered onto preweighed Whatman glass fiber filters (pore size 0.7µm). 20ml ethanol was used for extracting chlorophyll from each filter.

Calculation :

Chl *a* (ug/cm^2)= Chl*a* (ug/L) * ethanol (ml)* total volume of sample/volume of aliquots/surface area of tiles (cm^2); surface area of one tile (2*2inch)=25.8 cm^2

e.g. For sample 1-A, Chl *a* (ug/cm^2)= 329.5 (ug/L)*20ml*500ml/5ml/(2*25.8cm^2)=12.77

Table A6 Data of Chlorophyll *a* of periphyton community at the end of 7-day stream experiment

Stream treatments	Volume of aliquots (ml)	Total volume (ml)	Chl *a* (ug/L)	Chl *a* (ug/cm^2)
1-A	5	500	277.4	10.75
1-B	5	500	275.0	10.66
2-A	5	500	341.4	13.23
2-B	5	500	336.2	13.03
3-A	5	500	214.9	8.33
3-B	5	500	210.7	8.17
4-A	5	500	283.9	11.00
4-B	5	500	293.2	11.36
5-A	5	500	284.6	11.03
5-B	5	500	276.4	10.71
6-A	5	500	300.2	11.64
6-B	5	500	305.0	11.82

Note: calculation is the same as Table 5

Table A7 Data of ash-free dry mass of periphyton community at the start of 7-day stream experiment

Stream treatments	volume of aliquot (ml)	A=filter mass (mg)	B=DM+filter (mg)	C=DM(mg) =B-A	D(mg) =ash+filter	E=AFDM ($\mu g/cm^2$)
1-A	10	33.7	39.3	5.60	37.8	1453.49
1-B	10	33.8	39.1	5.30	37.6	1453.49
2-A	10	33.2	37.8	4.60	36.6	1162.79
2-B	10	33.7	38.3	4.60	37.1	1162.79
3-A	10	33.3	38.3	5.00	37.3	968.99
3-B	10	33.9	38.9	5.00	37.7	1162.79
4-A	10	33.2	37.7	4.50	36.4	1259.69
4-B	10	33.4	40.0	6.60	38.7	1259.69
5-A	10	33.2	42.0	8.80	40.4	1550.39
5-B	10	34.5	43.8	9.30	41.6	2131.78
6-A	10	33.0	40.1	7.10	38.7	1356.59
6-B	10	33.3	40.0	6.70	38.6	1356.59

Note: There are two replicates (A, B) for each stream treatment. Periphyton from 2 tiles were scraped by a hard-bristled toothbrush and diluted to 500ml, then 10ml aliquot of periphyton slurry filtered onto preweighed Whatman glass fiber filters (pore size 0.7µm).

Calculation:
AFDM($\mu g/cm^2$)=(DM-Ash)*total sample volume/aliquot volume/surface area of tiles
e.g. For sample 1-A,
AFDM($\mu g/cm^2$) =(39.3-37.8)mg*500ml/10ml/(25.8*2)cm^2=1.453mg/cm^2=1453µg/cm^2

Table A8 Data of ash-free dry mass of periphyton community at the end of 7-day stream experiment

Stream treatments	Volume of aliquots (ml)	A=filter mass (mg)	B=DM+filter (mg)	C=DM(mg) =B-A	D (mg)=ash+filter	E=AFDM (µg/cm^2)
1-A	10	33.45	38.53	5.08	37.30	1191.86
1-B	10	32.71	38.85	6.14	37.66	1153.10
2-A	10	33.58	40.6	7.02	39.24	1317.83
2-B	10	33.77	41.22	7.45	39.83	1346.90
3-A	10	32.95	39.87	6.92	38.46	1366.28
3-B	10	33.19	39.97	6.78	38.68	1250.00
4-A	10	33.41	41.17	7.76	39.73	1395.35
4-B	10	33.38	41	7.62	39.61	1346.90
5-A	10	33.15	39.19	6.04	38.02	1133.72
5-B	10	33.51	39.9	6.39	38.60	1259.69
6-A	10	33.23	39.31	6.08	38.05	1220.93
6-B	10	33.14	39.13	5.99	37.90	1191.86

Note: calculation is the same as Table 7

Table A9 Data of biomass-specific Cu concentration in periphyton after two-hour exposure in laboratory streams.

Stream treatments	Volume of aliquots (ml)	A=filter mass (mg)	B=DM+filter (mg)	C=DM(mg) =B-A	Cu (µg/L)	Cu (µg/mgDM)
1-A	10	33.4	36.4	3.0	0.094	0.006
1-B	10	32.8	35.6	2.8	1.225	0.087
2-A	10	33.9	38.3	4.4	0*	0.000
2-B	10	33.3	37.7	4.4	2.505	0.114
3-A	10	33	39.2	6.2	1.599	0.052
3-B	10	34.1	39.7	5.6	2.408	0.086
4-A	10	33.8	39.1	5.3	2.361	0.089
4-B	10	33	36.7	3.7	3.094	0.167
5-A	10	33.8	40.3	6.5	0*	0.000
5-B	10	33	39.8	6.8	0*	0.000
6-A	10	34	38.2	4.2	0*	0.000
6-B	10	33.2	37.7	4.5	0*	0.000

* under ICP-OES detection limit

Notes: There are two replicates (A, B) for each stream treatment. Periphyton from 2 tiles were scraped off and diluted to 500ml, then 10ml aliquot of periphyton slurry filtered onto preweighed glass fiber filters (pore size 0.7µm). After acid digestion, acid digested solution was diluted to 200ml with Milli-Q.

Calculation: Cu concentration in periphyton (µgCu/mgDM)= Cu (µg/L)*volume after dilution/DM
e.g. For sample 1-A,
Cu concentration in periphyton (µgCu/mgDM) =0.094(µg/L)*200ml/3.0mg=0.006(µg/mgDM)

Table A10 Data of biomass-specific Cu concentration in periphyton at the end of 7-day stream experiment

Stream treatments	Volume of aliquots(ml)	A=filter mass (mg)	B=DM+filter (mg)	C=DM(mg) =B-A	Cu (μg/L)	Cu (μg/mgDM)
1-A	10	33.45	39.32	5.87	0*	0.000
1-B	10	32.92	38.73	5.81	1.14	0.039
2-A	10	32.83	39.2	6.37	0.40	0.012
2-B	10	33.78	38.27	4.49	0*	0.000
3-A	10	32.86	38.62	5.76	6.00	0.208
3-B	10	33.28	39.87	6.59	7.22	0.219
4-A	10	33.37	40.78	7.41	6.46	0.174
4-B	10	33.82	41.05	7.23	6.26	0.173
5-A	10	33.81	39.92	6.11	0*	0.000
5-B	10	33.13	39.29	6.16	0*	0.000
6-A	10	33.03	39.11	6.08	0*	0.000
6-B	10	33	38.87	5.87	0*	0.000

Note: calculation is the same as Table 9
* Under ICP-OES detection limit

Table A11 Total Cu concentration of *H.azteca* tissue in periphyton-included treatments (PI) and periphyton-excluded treatments (PE).

Treatments	Nominal Cu conc. (µg /L)	Nominal P conc. (µg /L)	*H.azteca* weight (mg)	Acid digests Conc(µg /L)	Cu (µg/g tissue)
1(PI)	5	50	0.975	16.72	68.59
1(PE)	5	50	0.485	3.33	27.48
2(PI)	5	250	0.456	7.69	67.46
2(PE)	5	250	0.544	5.51	40.53
3(PI)	25	50	0.898	13.41	59.73
3(PE)	25	50	0.344	6.19	72.01
4(PI)	25	250	0.824	15.09	73.23
4(PE)	25	250	0.275	0.69	10.07
5(PI)	0	50	2.750	21.00	30.55
5(PE)	0	50	0.616	ND	ND
6(PI)	0	250	3.057	34.00	44.49
6(PE)	0	250	1.133	ND	ND

Note: 4ml acid digest in one vial

ND : not detectable

Calculation: Cu (µg/g tissue) = acid digested solution conc(µg /L)*volume of acid digested solution/ H.azteca weight (mg)

E.g. For sample 1(PI), Cu (µg/g tissue) = 16.72(µg /L)* 4ml/0.975mg tissue=68.59 (µg/g tissue)

Table A12 Growth and survival of *H.azteca* in periphyton-included treatments (PI).

Treatments	Nominal Cu conc. (μg /L)	Nominal P conc. (μg /L)	Growth (mm)	Survival
1-A	5	50	1.518	50%
1-B	5	50	1.419	80%
1-C	5	50	1.914	90%
1-D	5	50	1.489	100%
1-E	5	50	1.507	80%
2-A	5	250	0.903	100%
2-B	5	250	1.188	80%
2-C	5	250	0.978	100%
2-D	5	250	0.857	70%
2-E	5	250	1.016	80%
3-A	25	50	1.086	90%
3-B	25	50	0.880	80%
3-C	25	50	1.248	100%
3-D	25	50	0.952	70%
3-E	25	50	0.476	80%
4-A	25	250	0.745	70%
4-B	25	250	0.973	80%
4-C	25	250	0.595	80%
4-D	25	250	0.805	80%
4-E	25	250	1.517	100%
5-A	0	50	1.282	80%
5-B	0	50	0.986	100%
5-C	0	50	1.566	90%
5-D	0	50	1.255	80%
5-E	0	50	1.458	90%
6-A	0	250	1.314	100%
6-B	0	250	1.725	60%
6-C	0	250	1.647	100%
6-D	0	250	1.518	90%
6-E	0	250	1.171	100%

Note: There are five replicates (A-E) for each treatment.

Table A13 Growth and survival of *H.azteca* in periphyton-excluded treatments (PE).

Treatments	Nominal Cu conc. (µg /L)	Nominal P conc. (µg /L)	Growth (mm)	Survival
1-AW	5	50	0.953	90%
1-BW	5	50	0.753	90%
1-CW	5	50	0.925	80%
1-DW	5	50	0.725	90%
1-EW	5	50	0.703	80%
2-AW	5	250	0.435	90%
2-BW	5	250	0.979	90%
2-CW	5	250	0.789	50%
2-DW	5	250	0.802	70%
2-EW	5	250	0.801	70%
3-AW	25	50	1.344	100%
3-BW	25	50	0.852	100%
3-CW	25	50	0.653	70%
3-DW	25	50	0.703	100%
3-EW	25	50	0.384	60%
4-AW	25	250	0.881	100%
4-BW	25	250	0.493	70%
4-CW	25	250	0.471	100%
4-DW	25	250	0.803	100%
4-EW	25	250	0.612	80%
5-AW	0	50	0.644	100%
5-BW	0	50	0.502	100%
5-CW	0	50	0.32	100%
5-DW	0	50	0.527	100%
5-EW	0	50	0.547	100%
6-AW	0	250	0.361	100%
6-BW	0	250	0.272	90%
6-CW	0	250	0.175	100%
6-DW	0	250	0.027	100%
6-EW	0	250	0.04	100%

Note: There are five replicates (AW-EW) for each treatment.