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# Iron modulation of copper uptake and toxicity in a green alga (*Chlamydomonas reinhardtii*)

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## Abstract

Little attention has been paid to the role of essential trace elements on the toxicity of another element. In this work, we examined if low concentrations of essential elements (Co, Mn, Zn and Fe) modified the response of a freshwater green alga (*Chlamydomonas reinhardtii*) to copper. To do so, we followed cell growth over 72-h in exposure media where the essential element concentrations were manipulated. Among these elements, iron proved to have a strong impact on the cells' response to copper. The free $Cu^{2+}$ concentrations required to inhibit cellular growth by 50% (EC50) over 72-h decreased from 2 nM in regular Fe medium ($10^{-17.6}$ M $Fe^{3+}$) to 4 pM in low iron medium ($10^{-19.0}$ M $Fe^{3+}$); a 500-fold increase in toxicity. Moreover, at low $Cu^{2+}$ concentrations ($10^{-13.0}$ to $10^{-10.5}$ M), Cu uptake increased under low iron conditions but remain relatively stable under regular iron conditions. These results show clearly that iron plays a protective role against copper uptake and toxicity to *C. reinhardtii*. In freshwaters, iron is always abundant but the expected free iron concentrations in surface waters can vary between $10^{-14.0}$ to $10^{-20.0}$ M, depending on pH (e.g. when pH increases from 6 to 8). We conclude that copper toxicity in natural waters can be modulated by iron and that, in some conditions, the Biotic Ligand Model may need to be further developed to account for the influence of iron.

## Introduction

Understanding metal interactions with aquatic organisms is a key step to answer many questions about the nutritional importance of metals as well as their accumulation and toxicity. Research efforts in the last three decades of the 20$^{th}$ century have led to important paradigms such as the Free-Ion Activity Model (FIAM) and The Biotic Ligand Model (BLM).[1-4] These models are based on consensual data that show that metal accumulation and toxicity depend not on its total concentration in solution but on the free metal-ion concentration. In other words, ligands present in solution can complex metals and reduce their bioavailability to aquatic organisms. Thus metal speciation, i.e. distribution of their different metal species in solution, will be very important to determine harmful effects on aquatic organisms.[5, 6] While the FIAM was only considering metal speciation in the exposure solution, the BLM has shifted the focus on interactions at the solution-membrane interface, integrating competition among ions for binding. Concretely, interactions between ions in solution and cells take place at the cell surface with specific transport systems within the plasma membrane, which can lead to ion internalization. According to the BLM, the free metal ion $M^{z+}$ can bind to such a transport site but this binding can be inhibited by the presence of major cations such as $Ca^{2+}$, $Mg^{2+}$ or $H^{+}$. These competitive reactions can greatly reduce metal toxicity and many authors have contributed to formerly include these protective / antagonistic effects in the BLM at several levels of the food chain.[7-10] However, shortcomings or limitations of the BLM have been highlighted in some reviews which contribute to help develop or improve the BLM.[11-13] Among the possible limitations, the influence of essential micronutrients (e.g. Fe, Cu, Co, Mn, Zn) on metal uptake by freshwater organisms is not well known and is often assumed to be negligible compared to major cations (Ca, K, Mg, Na, H). Although major cations are more abundant (>1 mg/L) than micronutrients (<1 mg/L), this difference should be overcome by a greater affinity of the metals over major cations.[14] Research on this topic in freshwater systems is rare although a few publications have clearly indicated that their effects are not as negligible as was initially thought. For example, studies on metal adsorption onto bacteria, based on surface complexation modeling approaches, have provided insights on the incorporation of trace element

competition into the biotic ligand model.[15, 16] For freshwater algae, it was showed that Mn can affect Cd uptake and reduce its toxicity to *Scenedesmus vacuolatus*.[17] Similarily, it was observed that Zn can strongly affect Cd uptake and toxicity to *Chlamydomonas reinhardtii*.[18] In the latter case, the authors showed that low zinc concentrations could lead to an increase in zinc ion transporters which in turn can lead to an increase in cadmium uptake and toxicity. Moreover, the same authors found an abnormally high copper toxicity toward *C. reinhardtii* at a free copper concentration as low as $10^{-13}$ M when the exposure solution contained very low concentrations of essential elements.[18] The half-maximum effect concentration (EC50) previously reported for this species was $10^{-8.2}$ M $Cu^{2+}$.[19, 20] Thus, based on the intriguing observations of copper toxicity to *C. reinhardtii*, we hypothesized that the nutritive status of trace elements in the culture medium affects the sensitivity of this alga to Cu.[18] The role of iron was also more carefully looked into as copper and iron are closely involved in cellular metabolism. In fact, in many metabolic pathways copper and iron are used together or alternatively as heme, protein cofactor etc. suggesting that their cellular metabolisms are very closely linked.[21-24] We thus investigated the sensitivity of *C. reinhardtii* growth to copper and its accumulation in two different iron nutrition conditions: (*i*) in iron depleted media and (*ii*) in iron replete media.

## Experimental section

### *Biological material and culture medium*

The test alga, *Chlamydomonas reinhardtii*, was obtained from the Canadian Phycological Culture Centre (CPCC) of the University of Waterloo (Ontario, Canada). *C. reinhardtii* is a unicellular microalga of about ~ 5 μm in diameter, widely used as a biological model, notably for the study of interactions between metals and cells from an eco-physio-toxicological point of view.[18, 25] It is also genetically and biochemically well characterized and its genome has been fully sequenced.[25-29]

Algal cells were cultured in 100 mL of MHSM1 (Modified High Salt Medium) medium (Table S1) in 250 mL glass Erlenmeyer flasks.[30] A new autoclaved culture medium was inoculated once a week in order to maintain a good physiological state of the algae. MHSM1 medium was prepared from six stock solutions (Table S2) previously filtered and stored at 4°C. The ionic strength of the medium was adjusted to 22 $mEq \cdot L^{-1}$ with 1 M $NaNO_3$ solution and the pH was adjusted to 7.0 ± 0.1 with a 1 M NaOH solution. To buffer the solution pH, 3-N-morpholino-propanesulfonic acid (MOPS) 10 mM was used (see Table S1 for details of the solution composition). All manipulations were carried out axenically under a laminar flow hood and all culture flasks were autoclaved before their use. The cultured algae were placed on an orbital shaker (100 rpm) inside an environmental chamber (Conviron, CMP4030) maintained at a temperature of 20.0 ± 0.2 °C and the light intensity was maintained to 100 ± 10 µE or $\mu mol \cdot m^{-2} \cdot s^{-1}$ in order to obtain asynchronous algal cultures.

All vessels used in this study were soaked for 24 h in 15% (v/v) $HNO_3$ (ACS grade, Fisher Chemical) solution, washed five times with demineralized water, three times with ultrapure water, and dried under a class 100 laminar flow hood to avoid possible airborne particulates contamination.

***Exposure media and experimental designs***

The exposure media contained the same major nutrients as the MHSM1 culture medium, but instead of EDTA (ethylenediaminetetraacetic acid), metal concentrations were buffered by NTA (nitrilotriacetic acid). In our experiments, the concentrations of several essential elements were manipulated. In order to avoid co-variation of a free essential metal concentration upon the addition of another, the free $NTA^{3-}$ concentration was kept constant. This was achieved by the joint addition of an appropriate amount of NTA and metal as determined by thermodynamic calculations using MINEQL+ 5.0 speciation software as detailed in a previous publication.[18] For all experiments, algae previously cultured in MHSM1 medium, were collected, washed three times with the rinse solution (exposure medium without trace metals) to remove any EDTA, ligands or

trace metals associated with cell surface and transferred to exposure media for 72-h as described below.

Three series of copper exposures were carried out in triplicate. The first series was performed in a low metal medium (LM) in order to study the toxic effect of copper on cell growth when essential element concentrations are the lowest possible while high enough to sustain normal growth. The LM medium was designed to simulate an exposure medium in conditions where algae are at the limit of metal starvation, providing thus an opportunity to probe the impact of low essential elements on copper toxicity (Table S1). The second series of exposure was performed in four different media (LZn (Low Zinc), LMn (Low Manganese), LCo (Low Cobalt) and LFe (Low Iron); Tables S3 to 6) in which free essential metal concentrations were individually lowered to quantify their respective contribution in the observed copper uptake and toxicity (72-h cell growth). These exposure media were designed so that, in each case, only the metal of interest ($Zn^{2+}$, $Mn^{2+}$, $Co^{2+}$ or $Fe^{3+}$ respectively) was maintained at a low concentration with respect to the normal growth medium. This allowed us to investigate which one of these depleted essential metals has an impact on copper uptake and toxicity (the various treatments and their identification are presented in Tables S3, 4, 5 and 6). The third series of experiments were performed in High Metal media (HM; Table S7) and Low Fe media (LFe, Table S8). These allowed to quantify the effect of copper (uptake and toxicity) when all essential metals were maintained constant at the same free ion concentrations as in the standard culture medium (MHSM1), i.e. $10^{-7.1}$ M $Zn^{2+}$, $10^{-8.7}$ M $Co^{2+}$, $10^{-18}$ M $Fe^{3+}$ and $10^{-6}$ M $Mn^{2+}$ compared to a medium where free $Fe^{3+}$ concentration ($10^{-19}$ M $Fe^{3+}$) was lower. These exposure media allowed us to examine the cells' response under micronutrient-replete conditions (HM) compared to micronutrient-deplete conditions (LM) in order to test our working hypothesis. Free $Cu^{2+}$ concentrations ranged from $10^{-14.0}$ to $10^{-11.0}$ M in LM media and up to $10^{-8.5}$ M in HM media (see Tables S1 to 8 for details).

***Measurement process of cell density and copper accumulation***

Throughout the 72-h exposure, cell population density was measured at 0, 6, 12, 24, 48 and 72-h. Cell numbers, diameter and surface area were estimated with an electronic particle counter (Multisizer 3 Coulter Counter, 70-mm aperture; Beckman) after appropriate dilution in isotonic solution (Isoton® II). This allowed the construction of algal growth curves and for Cu uptake results to be normalized as a function of exposed surface area ($nmol \cdot m^{-2}$).

After the 72-h exposure period, algae were gently harvested by filtration which was carried out with a hand pump generating a low vacuum (10 cm Hg) in order to keep the algal cells intact. Two superimposed polycarbonate filter membranes (Millipore) of 2 μm porosity were used. The upper filter was used to harvest algae while the lower one was used to quantify potential Cu adsorption on the filter membrane. Aliquots of the filtrates were collected to determine copper concentration ($[Cu]_a$) remaining in the bulk solution after a given exposure period and for mass balance calculations (see below). After filtration, the algae were rinsed with 10 mL of rinse solution (simplified MHSM1 medium without trace elements) supplemented with EDTA ($10^{-4}$ M) for 10 min and then three times with 10 mL of the rinse solution. This step was designed to remove the Cu attached to the outer membrane of the algae and the remaining cell associated Cu was operationally defined as intracellular Cu.[31-33]

The two filter membranes were separately inserted in 50 mL propylene tubes with 5 mL of concentrated nitric acid (trace metal grade, Fisher) and 1 mL of hydrogen peroxide (Optima grade, Fisher). The tubes were then put in a digester at 95 °C and mineralized for 4 h. Then, 0.5 mL of hydrofluoric acid (ACS grade, Fisher) was added thereto and the tubes were placed again in the digester at 95 °C for an additional 2 h. After digestion, the volume of digestate was adjusted to 50 mL with ultrapure water (resistivity >18.2 MΩ cm; Nanopure grade) in order to reach 10% (v/v) of nitric acid matrix. All samples were stored at 4 °C and protected from light until copper analysis.

***Trace elements analysis and quality assurance and control***

Copper concentration in the samples were mainly analysed by ICP-MS (Inductively Coupled Plasma Mass Spectrometry, Thermo Scientific) for low Cu concentrations and ICP-AES (ICP Atomic Emission Spectrometry, Varian Vista AX) was used for higher trace element concentrations. Fisher Certified Reference Standards were used for the both analyses. Each sample concentration was determined in triplicate and the repeatability of ICP measurements was generally ≥ 97%. Analytical quality assurance (QA) and quality control (QC) were done using a certified reference material (CRM). For ICP analysis, single element standards (SCP Science) were used for calibration curve; multi-elemental certified standards (900-Q30-100, SCP Science) and a proficiency testing study for trace elements in water (#TE107-01, Environment Canada) were used as quality control for samples analysis, and the recoveries of each control material were 113 ± 1% and 95 ± 10%, respectively. Details of mass balances are provided in the supporting information (Tables S9 and 10); the average recovery of copper after the exposure is 83.0 ± 0.2 %. For acid digestions, the IAEA-413 algae material (International Atomic Energy Agency) was selected for quality control of experimental performance. The average recovery of Cu for the digestion process was 95.4 ± 3.5% (Table S11).

### *Statistical analysis*

All means are shown with standard deviation (± SD). Differences among treatments were analyzed by one-way analysis of variance (ANOVA). When significant differences were observed, means were compared using a t-test. The significant threshold was established at 0.05. Analyses were carried out using JMP Pro 13.0.0 software (SAS Institute, Riverside, CA, USA). All graphs and regression models were produced by using the Sigma Plot software (version 12.5). When measured values were pooled, error propagation calculations were performed.

## Results and Discussion

### *Combined and individual effects of essential trace metal on copper toxicity*

***Combined effect of trace elements***. While testing the effects of cationic metals on cadmium toxicity, Lavoie et al. noticed an abnormally high copper toxicity on algal growth in low [$Cu^{2+}$] ranged from $10^{-14}$ to $10^{-13}$ M.[18] They hypothesised that "low [$Cu^{2+}$] might exert greater toxicity in a growth medium with very low trace metal concentrations (but sufficiently high to sustain normal growth) than in metal-rich media because of a decrease in competing free essential metals". We thus set out to repeat this experiment and investigated if very low concentrations of copper could inhibit the growth of a freshwater green alga. To do so, we performed a long-term (72-h) exposure of *C. reinhardtii* to three very low $[Cu^{2+}]_{Free}$ ranging from $10^{-14}$ to $10^{-12}$ M in the LM medium which contained minimal concentrations of essential trace elements (Table S1). Results shown in Figure 1 indicate that the growth of *C. reinhardtii* was inhibited at the highest free Cu concentration tested ($10^{-12}$ M). Algal cells exposed to $10^{-13}$ and $10^{-14}$ M $Cu^{2+}$ showed a normal growth pattern while at $10^{-12}$ M, a much lower growth rate was observed following the lag phase which corresponded to the first 24 h. Indeed, the measured growth rates between 24 and 60-h (corresponding to the exponential growth phase) were 0.044 ± 0.002, 0.043 ± 0.002 and 0.017 ± 0.006 division $h^{-1}$ at $10^{-14}$, $10^{-13}$ and $10^{-12}$ M $Cu^{2+}$ respectively.

These results thus confirm that copper can be toxic to *C. reinhardtii* at very low concentrations when essential trace elements are present at low concentrations. In freshwaters, the focus is often on major cations ($Ca^{2+}$, $Mg^{2+}$ and $H^{+}$) as these have been shown to modify metal uptake and toxicity.[34, 35] Little attention has however been paid to essential trace metals that could also decrease uptake and toxicity of other metals, albeit this has been previously noticed for marine algae.[36-40] Therefore, we hypothesized that this enhanced sensitivity of algae to copper can be explained by the influence of the trace element nutritive status of the algae.[41, 42] We thus set out to figure out which micronutrient of interest is modulating the toxicity of Cu to *C. reinhardtii*.

***Individual effect of trace elements.*** To understand and quantify the contribution of each trace element present in the exposure solution to copper uptake and toxicity, we performed series of long-term (72-h) exposures of *C. reinhardtii* to $Cu^{2+}$ in different Low

Metal (LM) media (LCo, LFe, LMn and LZn) (see Tables S3 to 6). The results presented in Figure 2 show that, when only $Zn^{2+}$, $Mn^{2+}$ or $Co^{2+}$ was present at low concentration in the exposure media, no inhibition of algal growth by $Cu^{2+}$ was noticed after 72-h over the tested range of copper concentrations ($p>0.05$). On the other hand, when $Fe^{3+}$ was present at low concentration, $Cu^{2+}$ completely inhibited algal growth at $10^{-11}$ M ($p<0.0001$) (see Figure S1 in supplementary information for full growth curves). Thus, among these four trace elements, only $Fe^{3+}$ seems to modulate $Cu^{2+}$ uptake and toxicity to *C. reinhardtii*.

We then determined Cu toxicity thresholds (EC50) in both Fe replete and depleted cells (Fig. 3). Using the LFe medium ($[Fe^{3+}] = 10^{-19.0}$ M), an EC50 of only 4.2 pM [2.7 - 7.2; 95% confidence interval] was obtained. Using the HM medium ($[Fe^{3+}] = 10^{-17.6}$ M) we obtained a much higher EC50 (2.3 nM [1.8 – 2.9]) than in the LFe medium. This 500-fold difference indicates that copper can become much more toxic at very low Fe concentrations. Published copper EC50 toxicity data using the same algal species revealed toxicity thresholds of 6.0 ± 1.1 nM, a value close to the one we observed in the HM medium. To our knowledge, the lowest EC50 value based on free $Cu^{2+}$ for a unicellular alga previously published is 40.4 pM [39.6 – 41.2] obtained with *P. subcapitata* using natural surface waters.[9] This value is still one order of magnitude higher than what we observed in the low iron medium. Table S12 provides a summary of free $Cu^{2+}$ toxicity data found in the literature.

These results show clearly that iron plays a role in copper toxicity to *C. reinhardtii*. This assertion is consistent with the results of $Cu^{2+}$ accumulation inside the algae during their exposures to $Cu^{2+}$ under both iron depleted/replete conditions. According to the accumulation curves (Fig. 4), it appeared that, at low $Cu^{2+}$ concentrations ($\sim 10^{-13.0}$ to $10^{-10.5}$ M), Cu accumulation inside *C. reinhardtii* increases under iron-depleted conditions but remain constant under iron-replete conditions. In this range of $[Cu^{2+}]$, copper accumulation reached up to ~150 amol · cell$^{-1}$ representing a 6-fold increase compared to that observed for algal cells in iron-replete media. The impact of $Fe^{3+}$ on $Cu^{2+}$ uptake and toxicity in *C. reinhardtii* could be explained by two mechanisms: a competitive effect between $Fe^{3+}$ and $Cu^{2+}$ at the binding sites of metals on the cell surface as described in the BLM; and/or a feedback mechanism in which the low iron conditions trigger the

synthesis of additional Fe transporters.[43] Both mechanisms involve that Fe and Cu share a common transport system. The fact that these two metals are known to be metabolically linked is an argument in favor of this hypothesis.[21, 44-46] For example, Merchant et al. have found that multicopper oxidases are generally associated with permease or transporter for iron delivery across the membrane.[22] According to Kropat et al., in a copper-deficient situation, a blue copper protein (plastocyanin) can be replaced by cytochrome (Cyt) c6, a functionally equivalent heme (iron)-containing c-type Cyt.[47] Moreover, it was shown that Fe-deficient cells of *C. reinhardtii* exhibited greater copper reductase activity.[48] Additional work on higher plants also suggested that iron could have an impact on Cu uptake.[49-51] From a nutritional perspective, it can also be argued that the cells in conditions close to Fe limitations may become more susceptible to copper toxicity, despite normal growth at $[Cu^{2+}] \leq 3x10^{-13}$ M.

In the higher end of the concentration range ($[Cu^{2+}] \leq 10^{-10}$ M) which corresponds to the onset of growth inhibition for iron-replete cells, we observed that $Cu^{2+}$ accumulation increased up to 890 amol · $cell^{-1}$ (Fig. 4). This suggests that less internal copper is required in iron depleted cells to induce toxic effects compared to iron-replete cells. To further illustrate this point, we plotted dose-response curves and extracted EC50 values based on intracellular copper (Fig. 5). These clearly show that the internal dose required to inhibit growth by 50% is much lower for iron-depleted cells (104 amol/cell [93-115]) than for iron-replete cells (477 amol/cell [270-740]). In other words, iron can protect the cells from copper uptake but also from internal damage. This suggests that iron somehow modifies the internal handling of copper. The use of high resolution imaging techniques, metallomics or subcellular fractionation schemes could provide more information on how iron protects algal cells from copper toxicity.[52-54]

## Environmental implications

In freshwaters, as opposed to marine waters, iron is always abundant. The observed aqueous concentrations in surface waters are related to the watershed geochemistry. Due to dissolution and precipitation reactions involving iron hydroxides, iron mobility will be

295 strongly influenced by pH. Similarly, iron binding to dissolved organic matter will
296 contribute to the watershed transport of iron.[55] It follows that free iron concentrations in
297 the water column will be highly dependent on pH. At acidic pH, iron oxides become
298 more soluble and organic matter less binding due to proton competition. At circumneutral
299 pHs, iron becomes less soluble and stronger binding to organic matter occurs, driving
300 down the free iron concentrations. Lofts et al. estimated that free iron activity in
301 freshwaters decreased from $10^{-14}$ to $10^{-20}$ M when pH increased from 6 to 8.[56] In this
302 work, we showed that a decrease in free $Fe^{3+}$ from $10^{-17.6}$ to $10^{-19.0}$ resulted in a sharp
303 increase in copper sensitivity in a green alga. We thus predict that pH can influence
304 copper toxicity to unicellular algae in natural waters in three ways: i) through speciation
305 changes affecting free $Cu^{2+}$; ii) through competition between protons and $Cu^{2+}$ ions for
306 uptake; but also iii) from changes in ambient free $Fe^{3+}$ concentrations.

307

308 ASSOCIATED CONTENT

309 Supporting information

310 Details on the composition of all exposure media and their resulting speciation; Tables S1
311 to S8. Percent recoveries of total dissolved copper before and after exposure; Tables S9
312 and S10. Percent recoveries for standard material used during the mineralization
313 procedure; Table S11. Summary of free $Cu^{2+}$ toxicity data found in the literature; Table
314 S12. Growth curves in the presence of copper for the low Zn, Mn, Co or Fe experiments;
315 Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

316

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## References


1. Morel, F. M. M., *Principles and Applications of Aquatic Chemistry*. John Wiley & Sons, Inc.: Somerset, N.J., 1983; p 446.
2. Campbell, P. G. C., Interactions between trace metals and aquatic organisms: A critique of the free-ion activity model. In *Metal Speciation and Bioavailability in Aquatic Systems*, Tessier, A.; Turner, D. R., Eds. John Wiley & Sons: New York, NY, USA, 1995; pp 45-102.
3. Di Toro, D. M.; Allen, H. E.; Bergman, H. L.; Meyer, J. S.; Paquin, P. R.; Santore, R. C., Biotic ligand model of the acute toxicity of metals. 1. Technical basis. *Environ Toxicol Chem* **2001,** *20*, (10), 2383-2396.
4. Paquin, P.; Gorsuch, J.; Apte, S.; Batley, G. E.; Bowles, K. C.; Campbell, P. G. C.; Delos, C. G.; Di Toro, D. M.; Dwyer, R. L.; Galvez, F.; Gensemer, R. W.; Goss, G. G.; Hogstrand, C.; Janssen, C. R.; McGeer, J. C.; Naddy, R. B.; Playle, R. C.; Santore, R. C.; Schneider, U.; Stubblefield, W. A.; Wood, C. M.; Wu, K. B., The biotic ligand model: a historical overview. *Comp Biochem Physiol C* **2002,** *133*, (1-2), 3-35.
5. Janssen, C. R.; Heijerick, D. G.; De Schamphelaere, K. A. C.; Allen, H. E., Environmental risk assessment of metals: tools for incorporating bioavailability. *Environ Int* **2003,** *28*, (8), 793-800.
6. Batley, G. E.; Apte, S. C.; Stauber, J. L., Speciation and bioavailability of trace metals in water: Progress since 1982. *Aust J Chem* **2004,** *57*, (10), 903-919.
7. De Schamphelaere, K. A. C.; Janssen, C. R., A biotic ligand model predicting acute copper toxicity for *Daphnia magna*: The effects of calcium, magnesium, sodium, potassium, and pH. *Environ Sci Technol* **2002,** *36*, (1), 48-54.
8. Pagenkopf, G. K., Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH and water hardness. *Environ Sci Technol* **1983,** *17*, (6), 342-347.
9. De Schamphelaere, K. A. C.; Vasconcelos, F. M.; Heijerick, D. G.; Tack, F. M. G.; Delbeke, K.; Allen, H. E.; Janssen, C. R., Development and field validation of a predictive copper toxicity model for the green alga *Pseudokirchneriella subcapitata*. *Environ Toxicol Chem* **2003,** *22*, (10), 2454-2465.
10. Gensemer, R. W.; Naddy, R. B.; Stubblefield, W. A.; Hockett, J. R.; Santore, R. C.; Paquin, P., Evaluating the role of ion composition on the toxicity of copper to *Ceriodaphnia dubia* in very hard waters. *Comp Biochem Physiol C* **2002,** *133*, (1-2), 87.
11. Campbell, P. G. C.; Errécalde, O.; Fortin, C.; Hiriart-Baer, V. P.; Vigneault, B., Metal bioavailability to phytoplankton-applicability of the biotic ligand model. *Comp Biochem Physiol C* **2002,** *133*, (1-2), 189-206.
12. Niyogi, S.; Wood, C. M., Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. *Environ Sci Technol* **2004,** *38*, (23), 6177-6192.
13. Slaveykova, V. I.; Wilkinson, K. J., Predicting the bioavailability of metals and metal complexes: Critical review of the biotic ligand model. *Environ Chem* **2005,** *2*, (1), 9-24.

14. Stumm, W.; Morgan, J., *Aquatic Chemistry: Chemical Equilibria and Rates in Natural Waters*. Third ed.; John Wiley and Sons: New York, 1996; p 1022.
15. Fein, J. B., Advanced biotic ligand models: Using surface complexation modeling to quantify metal bioavailability to bacteria in geologic systems. *Chem Geol* **2017,** *464*, 127-136.
16. Duval, J. F., Coupled metal partitioning dynamics and toxicodynamics at biointerfaces: a theory beyond the biotic ligand model framework. *Phys Chem Chem Phys* **2016,** *18*, (14), 9453-69.
17. Töpperwien, S.; Behra, R.; Sigg, L., Competition among zinc, manganese, and cadmium uptake in the freshwater alga *Scenedesmus vacuolatus*. *Environ Toxicol Chem* **2007,** *26*, (3), 483-490.
18. Lavoie, M.; Fortin, C.; Campbell, P. G. C., Influence of essential elements on cadmium uptake and toxicity in a unicellular green alga: The protective effect of trace zinc and cobalt concentrations. *Environ Toxicol Chem* **2012,** *31*, (7), 1445-1452.
19. Stoiber, T. L.; Shafer, M. M.; Armstrong, D. E., Differential effects of copper and cadmium exposure on toxicity endpoints and gene expression in *Chlamydomonas reinhardtii*. *Environ Toxicol Chem* **2010,** *29*, (1), 191-200.
20. Stoiber, T. L.; Shafer, M. M.; Armstrong, D. E., Relationships between surface-bound and internalized copper and cadmium and toxicity in *Chlamydomonas reinhardtii*. *Environ Toxicol Chem* **2012,** *31*, (2), 324-335.
21. La Fontaine, S.; Quinn, J.; Merchant, S., Comparative analysis of copper and iron metabolism in photosynthetic eukaryotes vs yeast and mammals. In *Handbook of Copper Pharmacology and Toxicology*, Springer: 2002; pp 481-502.
22. Merchant, S. S.; Allen, M. D.; Kropat, J.; Moseley, J. L.; Long, J. C.; Tottey, S.; Terauchi, A. M., Between a rock and a hard place: trace element nutrition in *Chlamydomonas*. *Biochim Biophys Acta* **2006,** *1763*, (7), 578-594.
23. Schoffman, H.; Lis, H.; Shaked, Y.; Keren, N., Iron–nutrient interactions within phytoplankton. *Front Plant Sci* **2016,** *7*, 1223.
24. La Fontaine, S.; Quinn, J. M.; Nakamoto, S. S.; Page, M. D.; Göhre, V.; Moseley, J. L.; Kropat, J.; Merchant, S., Copper-dependent iron assimilation pathway in the model photosynthetic eukaryote *Chlamydomonas reinhardtii*. *Eukaryot Cell* **2002,** *1*, (5), 736-757.
25. Hanikenne, M., *Chlamydomonas reinhardtii* as a eukaryotic photosynthetic model for studies of heavy metal homeostasis and tolerance. *New Phytol* **2003,** *159*, (2), 331-340.
26. Kathir, P.; LaVoie, M.; Brazelton, W. J.; Haas, N. A.; Lefebvre, P. A.; Silflow, C. D., Molecular map of the *Chlamydomonas reinhardtii n*uclear genome. *Eukaryot Cell* **2003,** *2*, (2), 362-379.
27. Merchant, S. S.; Prochnik, S. E.; Vallon, O.; Harris, E. H.; Karpowicz, S. J.; Witman, G. B.; Terry, A.; Salamov, A.; Fritz-Laylin, L. K.; Maréchal-Drouard, L.; Marshall, W. F.; Qu, L.-H.; Nelson, D. R.; Sanderfoot, A. A.; Spalding, M. H.; Kapitonov, V. V.; Ren, Q.; Ferris, P.; Lindquist, E.; Shapiro, H.; Lucas, S. M.; Grimwood, J.; Schmutz, J.; Cardol, P.; Cerutti, H.; Chanfreau, G.; Chen, C.-L.; Cognat, V.; Croft, M. T.; Dent, R.; Dutcher, S.; Fernández, E.; Ferris, P.; Fukuzawa, H.; González-Ballester, D.; González-Halphen, D.; Hallmann, A.; Hanikenne, M.; Hippler, M.;

Inwood, W.; Jabbari, K.; Kalanon, M.; Kuras, R.; Lefebvre, P. A.; Lemaire, S. D.; Lobanov, A. V.; Lohr, M.; Manuell, A.; Meier, I.; Mets, L.; Mittag, M.; Mittelmeier, T.; Moroney, J. V.; Moseley, J.; Napoli, C.; Nedelcu, A. M.; Niyogi, K.; Novoselov, S. V.; Paulsen, I. T.; Pazour, G.; Purton, S.; Ral, J.-P.; Riaño-Pachón, D. M.; Riekhof, W.; Rymarquis, L.; Schroda, M.; Stern, D.; Umen, J.; Willows, R.; Wilson, N.; Zimmer, S. L.; Allmer, J.; Balk, J.; Bisova, K.; Chen, C.-J.; Elias, M.; Gendler, K.; Hauser, C.; Lamb, M. R.; Ledford, H.; Long, J. C.; Minagawa, J.; Page, M. D.; Pan, J.; Pootakham, W.; Roje, S.; Rose, A.; Stahlberg, E.; Terauchi, A. M.; Yang, P.; Ball, S.; Bowler, C.; Dieckmann, C. L.; Gladyshev, V. N.; Green, P.; Jorgensen, R.; Mayfield, S.; Mueller-Roeber, B.; Rajamani, S.; Sayre, R. T.; Brokstein, P.; Dubchak, I.; Goodstein, D.; Hornick, L.; Huang, Y. W.; Jhaveri, J.; Luo, Y.; Martínez, D.; Ngau, W. C. A.; Otillar, B.; Poliakov, A.; Porter, A.; Szajkowski, L.; Werner, G.; Zhou, K.; Grigoriev, I. V.; Rokhsar, D. S.; Grossman, A. R., The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science (New York, N.Y.)* **2007,** *318*, (5848), 245-250.

28. Harris, E. H., *The Chlamydomonas Sourcebook: Introduction to Chlamydomonas and its Laboratory Use*. Elsevier Science: 2009.
29. Pröschold, T.; Harris, E. H.; Coleman, A. W., Portrait of a species: *Chlamydomonas reinhardtii*. *Genetics* **2005,** *170*, (4), 1601-1610.
30. Fortin, C.; Campbell, P. G. C., Silver uptake by the green alga *Chlamydomonas reinhardtii* in relation to chemical speciation: influence of chloride. *Environ Toxicol Chem* **2000,** *19*, (11), 2769-2778.
31. Knauer, K.; Behra, R.; Sigg, L., Adsorption and uptake of copper by the green alga *Scenedesmus subspicatus* (Chlorophyta). *J Phycol* **1997,** *33*, (4), 596-601.
32. Mehta, S. K.; Tripathi, B. N.; Gaur, J. P., Influence of pH, temperature, culture age and cations on adsorption and uptake of Ni by *Chlorella vulgaris*. *Eur J Protistol* **2000,** *36*, (4), 443-450.
33. Hassler, C. S.; Slaveykova, V. I.; Wilkinson, K. J., Discriminating between intra- and extracellular metals using chemical extractions. *Limnol Oceanogr Methods* **2004,** *2*, 237-247.
34. Deleebeeck, N. M. E.; De Schamphelaere, K. A. C.; Janssen, C. R., A bioavailability model predicting the toxicity of nickel to rainbow trout (*Oncorhynchus mykiss*) and fathead minnow (*Pimephales promelas*) in synthetic and natural waters. *Ecotoxicol Environ Saf* **2007,** *67*, (1), 1-13.
35. François, L.; Fortin, C.; Campbell, P. G. C., pH modulates transport rates of manganese and cadmium in the green alga *Chlamydomonas reinhardtii* through non-competitive interactions: Implications for an algal BLM. *Aquat Toxicol* **2007,** *84*, (2), 123-132.
36. Foster, P. L.; Morel, F. M. M., Reversal of cadmium toxicity in a diatom: An interaction between cadmium activity and iron. *Limnol Oceanogr* **1982,** *27*, (4), 745-752.
37. Harrison, G. I.; Morel, F. M. M., Antagonism between cadmium and iron in the marine diatom *Thalassiosira weissflogii*. *J Phycol* **1983,** *19*, (4), 495-507.
38. Sunda, W. G.; Huntsman, S. A., Antagonisms between cadmium and zinc toxicity and manganese limitation in a coastal diatom. *Limnol Oceanogr* **1996,** *41*, (3), 373-387.

39. Sunda, W. G.; Huntsman, S. A., Interactions among $Cu^{2+}$, $Zn^{2+}$, and $Mn^{2+}$ in controlling cellular Mn, Zn, and growth rate in the coastal alga *Chlamydomonas*. *Limnol Oceanogr* **1998,** *43*, (6), 1055-1064.
40. Lane, E. S.; Jang, K.; Cullen, J. T.; Maldonado, M. T., The interaction between inorganic iron and cadmium uptake in the marine diatom *Thalassiosira oceanica*. *Limnol Oceanogr* **2008,** *53*, (5), 1784-1789.
41. Burkhead, J. L.; Gogolin Reynolds, K. A.; Abdel-Ghany, S. E.; Cohu, C. M.; Pilon, M., Copper homeostasis. *New Phytol* **2009,** *182*, (4), 799-816.
42. Georgatsou, E.; Mavrogiannis, L. A.; Fragiadakis, G. S.; Alexandraki, D., The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. *J Biol Chem* **1997,** *272*, (21), 13786-13792.
43. Raven, J. A., Nutrient transport in microalgae. In Rose, A. H.; Morris, J. G., Eds. Academic Press: New York, NY, USA, 1980; pp 47-226.
44. Arredondo, M.; Núñez, M. T., Iron and copper metabolism. *Mol Aspects Med* **2005,** *26*, (4), 313-327.
45. Taylor, A. B.; Stoj, C. S.; Ziegler, L.; Kosman, D. J.; Hart, P. J., The copper-iron connection in biology: Structure of the metallo-oxidase Fet3p. *Proc Nat Acad Sci* **2005,** *102*, (43), 15459-15464.
46. Hill, K. L.; Hassett, R.; Kosman, D.; Merchant, S., Regulated copper uptake in *Chlamydomonas reinhardtii* in response to copper availability. *Plant Physiol* **1996,** *112*, (2), 697-704.
47. Kropat, J.; Gallaher, S. D.; Urzica, E. I.; Nakamoto, S. S.; Strenkert, D.; Tottey, S.; Mason, A. Z.; Merchant, S. S., Copper economy in *Chlamydomonas*: Prioritized allocation and reallocation of copper to respiration vs. photosynthesis. *Proc Nat Acad Sci* **2015,** *112*, (9), 2644-2651.
48. Herbik, A.; Bolling, C.; Buckhout, T. J., The involvement of a multicopper oxidase in iron uptake by the green algae *Chlamydomonas reinhardtii*. *Plant Physiol* **2002,** *130*, (4), 2039-2048.
49. Krämer, U.; Talke, I. N.; Hanikenne, M., Transition metal transport. *FEBS Letters* **2007,** *581*, (12), 2263-2272.
50. Schmidt, W.; Bartels, M.; Tittel, J.; Fuhner, C., Physiological effects of copper on iron acquisition processes in *Plantago*. *New Phytol* **1997,** *135*, (4), 659-666.
51. Chen, Y.; Shi, J.; Tian, G.; Zheng, S.; Lin, Q., Fe deficiency induces Cu uptake and accumulation in *Commelina communis*. *Plant Sci* **2004,** *166*, (5), 1371-1377.
52. Liu, Q.; Zhou, L.; Liu, F.; Fortin, C.; Tan, Y.; Huang, L.; Campbell, P. G. C., Uptake and subcellular distribution of aluminum in a marine diatom. *Ecotoxicol Environ Saf* **2019,** *169*, 85-92.
53. Lavoie, M.; Le Faucheur, S.; Fortin, C.; Campbell, P. G. C., Cadmium detoxification strategies in two phytoplankton species: Metal binding by newly synthesized thiolated peptides and metal sequestration in granules. *Aquat Toxicol* **2009,** *92*, (2), 65-75.
54. Slaveykova, V. I.; Guignard, C.; Eybe, T.; Migeon, H. N.; Hoffmann, L., Dynamic NanoSIMS ion imaging of unicellular freshwater algae exposed to copper. *Anal Bioanal Chem* **2009,** *393*, (2), 583-589.

55. Tipping, E.; Rey-Castro, C.; Bryan, S. E.; Hamilton-Taylor, J., Al(III) and Fe(III) binding by humic substances in freshwaters, and implications for trace metal speciation. *Geochim Cosmochim Acta* **2002,** *66*, (18), 3211-3224.
56. Lofts, S.; Tipping, E.; Hamilton-Taylor, J., The chemical speciation of Fe(III) in freshwaters. *Aquat Geochem* **2008,** *14*, (4), 337-358.

Figure 1: Growth of *C. reinhardtii* cell population over time in a low metal medium in which essential element concentrations are the lowest possible while high enough to sustain normal growth. Only the free copper concentration was varied (N=3).

Figure 2: Growth of *C. reinhardtii* after 72-h of exposure to four different concentrations of free ionic copper ($10^{-14}$, $10^{-13}$, $10^{-12}$ and $10^{-11}$ M) in four exposure media. In each medium, the concentration of one essential element was manipulated (Zn, Mn, Co and Fe). Each panel shows the cell density after 72-h as a function of $Cu^{2+}$ concentration. Error bars are the standard deviations of three replicates. In the low Fe medium, the growth difference between copper treatments is significant (ANOVA; $p<0.001$) (See Tables S3 to 6 for the total and free concentrations of each element).

Figure 3: Relative cell yield ($\rho/\rho_0$) after 72-h as a function of exposure copper concentrations in low (○, LFe) and high iron media (●, HM). Error bars represent the standard deviations of two or three replicates of algal cultures. Half-maximal effective concentrations (EC50) are given with their respective 95% confidence intervals.

Figure 4: Accumulation of Cu in *C. reinhardtii* after 72-h of exposure as a function of free copper concentrations in low (○, LFe) and high iron media (●, HM). Error bars are the standard deviations of two or three replicates.

Figure 5: Dose-response curves showing relative cell populations after 72-h as a function of intracellular copper accumulation (in amol/cell) in low (○, LFe) and high iron media (●, HM) exposed cells. The error bars represent standard deviations of the mean of two to three replicates.

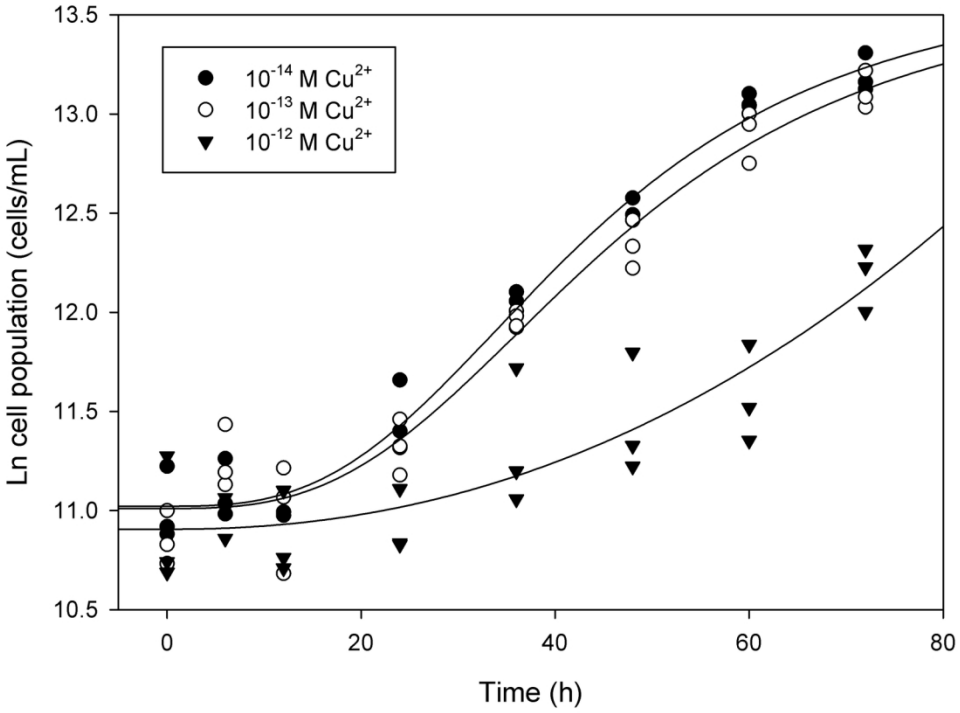


Figure 1

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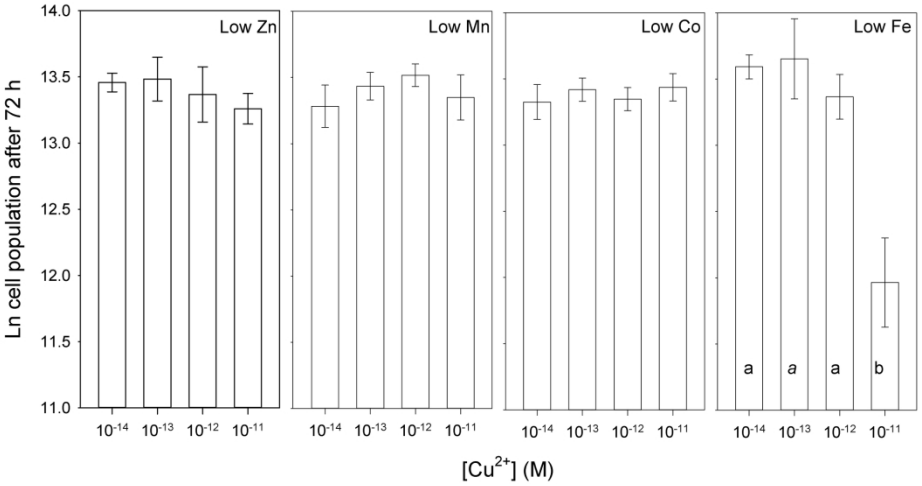


Figure 2

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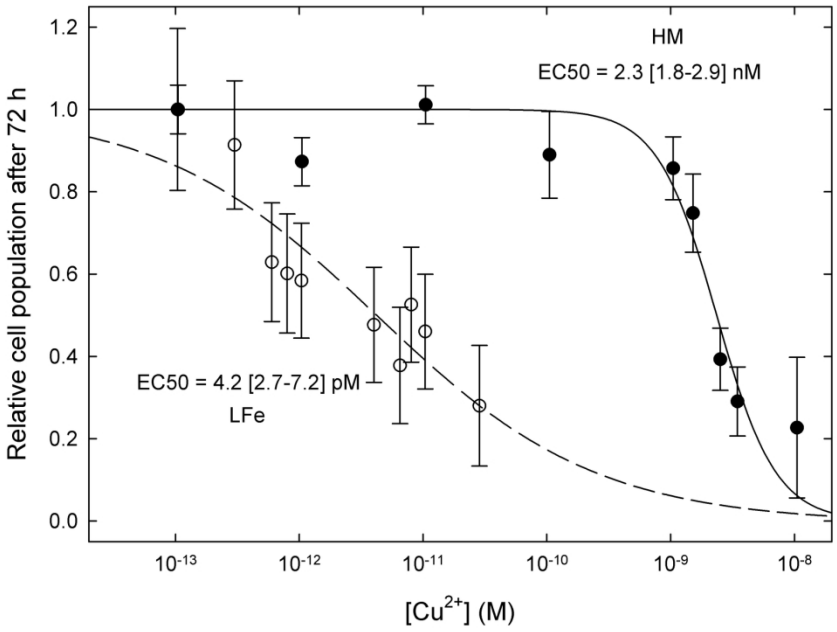


Figure 3

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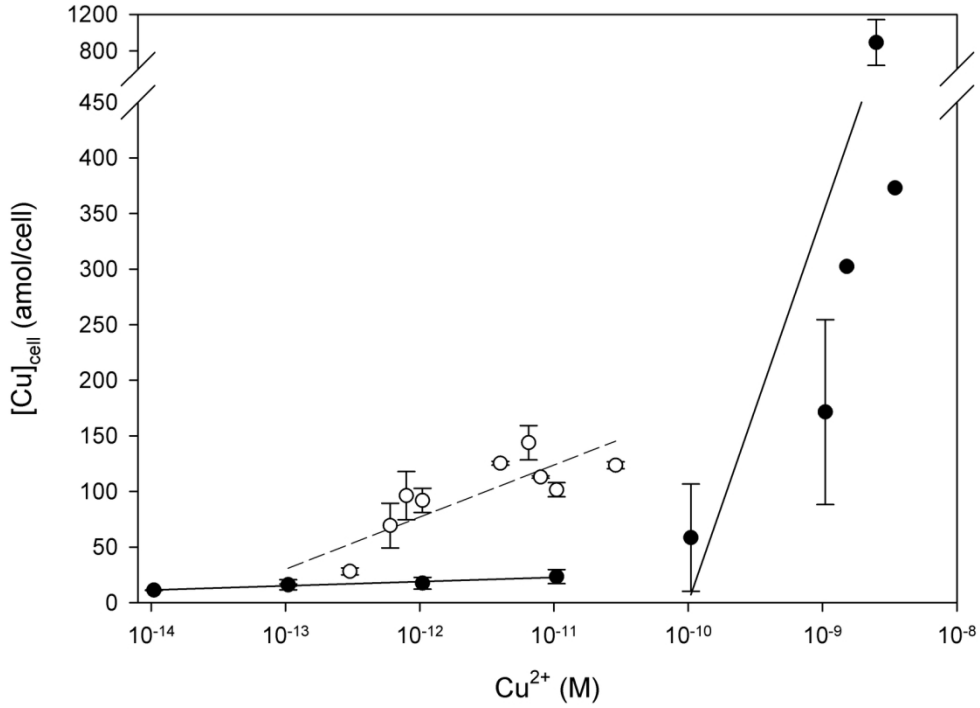


Figure 4

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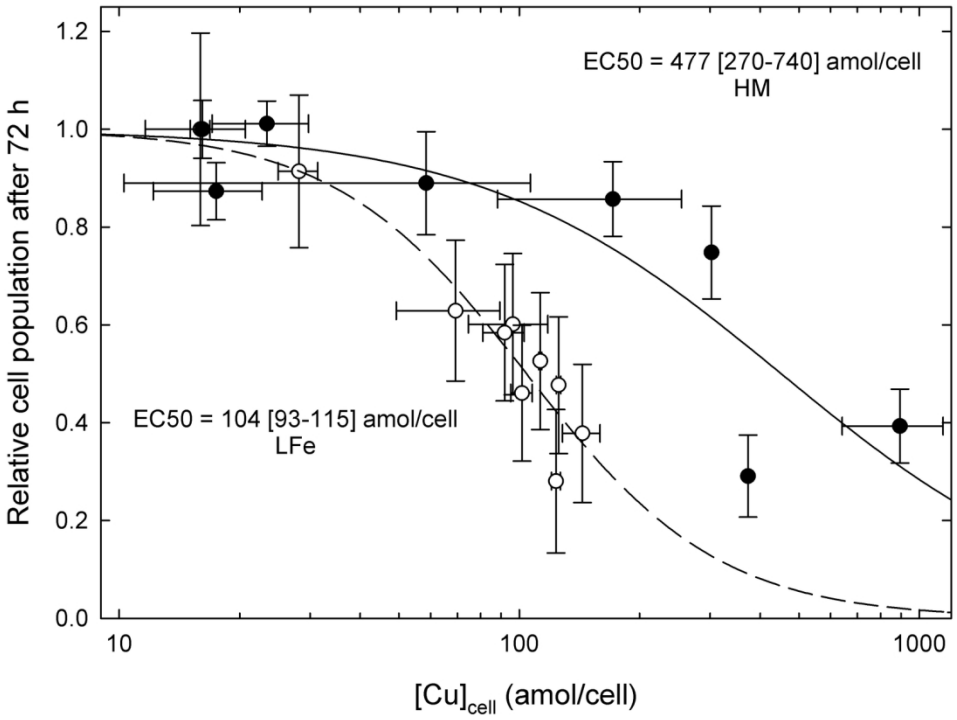


Figure 5

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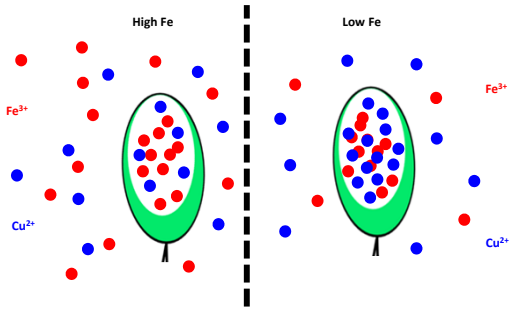


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