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## Isotopic insights into biological regulation of zinc in contaminated systems

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### Abstract

Aquatic organisms use a variety of biogeochemical reactions to regulate essential and non-essential trace metals. Many of these mechanisms can lead to isotopic fractionation, thus measurement of metal isotopes may yield insights into the processes by which organisms respond to metal exposure. We illustrate these concepts with two case studies, one involving an intra- and the other an extra-cellular mechanism of Zn sequestration. In the first study, the mayfly *Neocloeon triangulifer* was grown in the laboratory, and fed a diet of Zn-doped diatoms at Zn levels exceeding the requirements for normal mayfly life functions. The *N. triangulifer* larvae consumed the diatoms and retained their Zn isotopic signature. Upon metamorphosis, the subimago life stage lost Zn mass either in the exuvia or by excretion, and the Zn retained was isotopically enriched. Thus, Zn uptake is non-fractionating, but Zn regulation favors the lighter isotope. Thus the Zn remaining in the subimago was isotopically heavier. In the second study, Zn was adsorbed on the cell walls and exopolysaccharide secretions of cyanobacteria, which favored the heavier Zn isotope. Continued adsorption eventually resulted in nucleation and biomineralization of hydrozincite  $\{Zn_3(CO_3)_2(OH)_6\}$ . These case studies demonstrate the utility of Zn isotopes to provide insights into how aquatic insects respond to metal exposure.

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### 1. Introduction

Numerous authors have investigated the effects of various trace metals on aquatic organisms<sup>1-3</sup>, and found a multitude of variables that affects the toxicity of each metal. Metal speciation and bioavailability, the rates of uptake,

elimination and detoxification, and the unique sensitivity of receiving organisms, influence the toxicological response. Organisms survive when they can avoid, excrete, or accommodate metals in their bodies by some mechanism, at a combined rate exceeding uptake<sup>1</sup>. These compensatory mechanisms, many of which are kinetically controlled reactions, may leave a signature by fractionating metal isotopes. As a result, isotopic analyses may reveal previously unknown details about these biochemical reactions.

### Nomenclature

$\delta$  isotope ratio ( $^{66}\text{Zn}/^{64}\text{Zn}$ ) of a sample ( $R_{\text{spl}}$ ) relative to a standard ( $R_{\text{std}}$ - JMC 3-0749 Lyon), expressed in parts per thousand (‰), and given by the formula:  $\delta = (R_{\text{spl}}/R_{\text{std}}-1) * 1000$

#### 1.1. Methods

For the *Neocloeon triangulifer* experiment, insects were grown in the laboratory in a Zn-free solution, but fed diatoms that were grown in a Zn-spiked medium of known isotopic composition. At the end of the growing period for the insects, dried bodies were weighed and digested in a mixture of  $\text{HNO}_3$  and  $\text{H}_2\text{O}_2$  for total Zn and Zn isotopic analyses. For the hydrozincite study, water and solid samples were collected in the field<sup>5</sup> and preserved for total Zn and Zn isotopic analyses. All samples were prepared for Zn isotope analyses by established methods<sup>6</sup>; Zn isotopic analyses were performed on a Nu Instruments HR multicollector ICP-MS, with standard-sample-standard bracketing. In both cases, analytical quality was monitored and maintained through the use of blanks, standards, procedural-duplicate samples, and replicate analyses of the same sample. For  $\delta^{66}\text{Zn}$ , analytical uncertainty, based on numerous replicated analyses, is within  $\pm 0.1\%$  ( $2\sigma$ ).

## 2. Results and Discussion

Figure 1 is a conceptual model for aquatic organism responses to metal exposure. A cascade of biologically mediated processes can be initiated as the organisms are exposed to trace metals; these processes may be extra- or intra-cellular. The *N. triangulifer* experiment represents a series of intra-cellular processes, while the hydrozincite biomineralization represents extra-cellular processes.

#### 2.1. Uptake of dietary Zn by *Neocloeon triangulifer*

The primary pathway for Zn uptake by the *N. triangulifer* was through their diet of diatoms. As seen in Fig. 2, the Zn concentration in the larvae (squares) is greater than that of the diatoms, but the Zn isotope ratios (circles) are identical, indicating quantitative uptake and retention of Zn by the *N. triangulifer*. In this case, ‘quantitative uptake’ implies that the rate of ingestion greatly exceeds the rate of excretion, consistent with previous studies<sup>7</sup>. Upon metamorphosis to the subimago (flying pre-adult) stage, the mayflies lose appreciable mass of Zn, and the Zn lost is isotopically light relative to that remaining in their bodies. The fact that Zn is lost in the metamorphosis from larval to subimago stage implies that the larvae consumed Zn in excess of that which is metabolically essential. Two possibilities may explain this result: the process by which metabolically essential Zn is captured favors the heavy isotopes, or the process by which Zn is sequestered in a non-toxic form favors the lighter isotope.

If the uptake of metabolic (essential) Zn favors the heavy isotope, we might expect to see fractionation of Zn between the diatoms and the larvae, which is not the case. Thus the second scenario, where sequestration (detoxification) mechanisms of Zn favor the light isotope, seems more likely. Sequestration would likely be a kinetically controlled process, governed by the relative rates of Zn uptake by the larvae (rate of eating), rate of excretion (either near-zero<sup>7</sup> or a non-fractionating process), and rate(s) of detoxification mechanism(s). Although the difference in Zn isotope ratio is observed in the metamorphosis step from larvae to subimago, the actual Zn fractionation must occur within the bodies of the larvae, as they differentiate between metabolically useful and excess Zn. The excess Zn is then shed during metamorphosis, either in the exuvia or by excretion<sup>8</sup>.

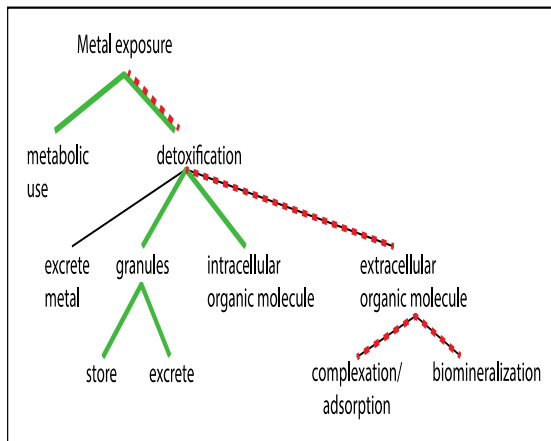


Fig. 1. Flowchart depicting mechanisms by which an organism may react to excess metal exposure. Green lines represent possible pathways taken by *N. triangulifer*; dashed red lines are pathways taken by bacteria in hydrozincite biomineralization.

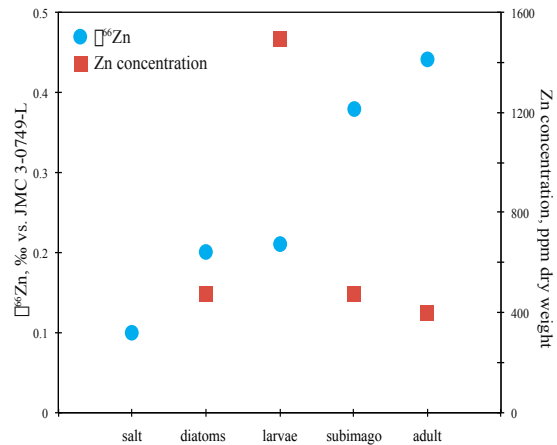


Fig. 2. Average Zn whole-body concentration (squares) and isotopic ratio (circles) for the various components and life stages of the experiment involving *N. triangulifer*. [salt, n=1; diatoms n=2; larvae and subimago, n=5, adults, n=2]

## 2.2. Hydrozincite biomineralization by microalgae and cyanobacteria

In the Rio Naracauli in SW Sardinia, Italy, very high concentrations of Zn (10s of mg/L) with near-neutral pH are the result of drainage from Zn-rich mine wastes, which also are rich in carbonate minerals such as calcite. Because of the pH, concentrations of other metals such as Fe, Cu, Ni, and Co are very low- usually <100  $\mu\text{g/L}$ . In the upper part of Rio Naracauli, the streambed is covered seasonally with a fine precipitate of hydrozincite [ $\text{Zn}_5(\text{CO}_3)_2(\text{OH})_6$ ]. However, within the stream water, even though hydrozincite is supersaturated<sup>9</sup>, it does not precipitate directly from solution, probably due to a very high nucleation energy required to form such a complex mineral lattice. Rather the hydrozincite is observed to form in contact with a bacterial flora including a microalga (*Chlorella* sp.) and a cyanobacterium (*Scytonema* sp.)<sup>10</sup>.

As seen in Fig. 3A, the Zn concentration decreases proceeding downstream along the Rio Naracauli, as does the value of the isotopic ratio  $\delta^{66}\text{Zn}$ ; both of these results are due to the biomineralization of hydrozincite<sup>5</sup>. Investigations of paired samples of stream water and co-formed hydrozincite revealed that Zn in hydrozincite was always around +0.35‰ heavier than the water from which it formed, which was attributed to adsorption of Zn onto the bacterial surface as a precursor to mineral formation<sup>5</sup>. This initial adsorption step precedes the organization of the components of hydrozincite into the crystal lattice. This latter step- nucleation- occurs as a result of an extreme microenvironment at the bacterial surface, with greatly enriched alkalinity relative to the bulk solution<sup>11</sup>. Thus, the extracellular process (Fig. 3) involved in this biomineralization favors the heavy Zn isotope, consistent with other studies that have shown that adsorption onto biological surfaces enriches Zn with respect to the bulk solution<sup>12,13</sup>.

## 3. Summary

Zinc isotopes are fractionated during biological regulation, and the direction and magnitude of the fractionation may yield clues to the mechanisms involved. The two case studies that were used here to illustrate this concept involve a series of kinetically controlled intracellular processes of uptake and detoxification, and an extracellular process of biomineralization that probably occurs close to equilibrium. In both cases the magnitude of isotope fractionation is a few tenths of a per mil, consistent with numerous literature studies that show that Zn isotopes fractionate within a relatively narrow range<sup>12</sup>. In this study, we have been able to infer intra- vs. extracellular processes based on features of experimental design, as well as using measurements of total Zn and Zn isotopes, and other data such as microscopic evidence.

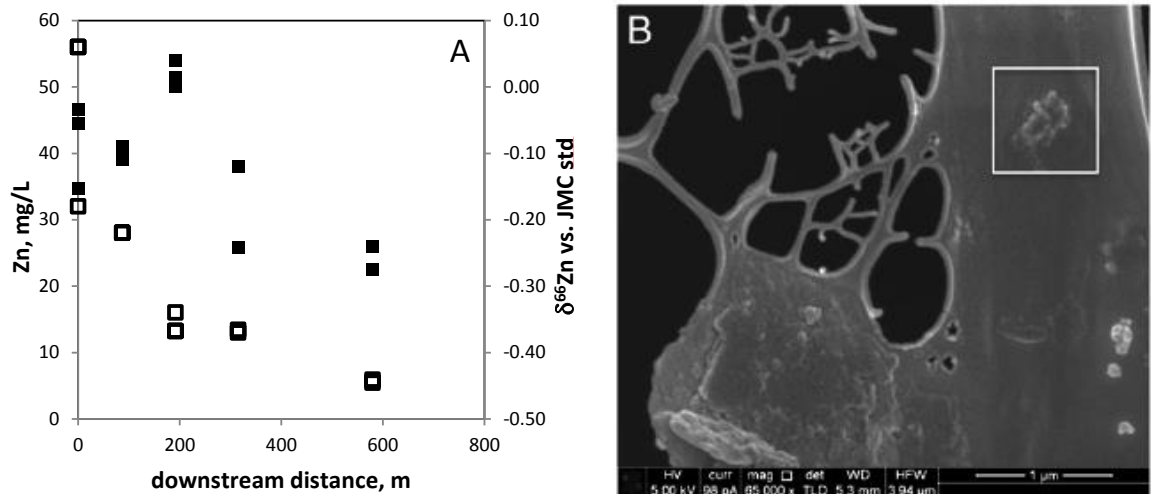


Fig. 3. A) Concentration and Zn isotope signature of water in the Rio Naracauli as a function of distance downstream. As hydrozincite precipitates, favoring the heavier isotope of Zn, both the Zn concentration (open symbols) and the value of  $\delta^{66}\text{Zn}$  (solid symbols) decrease. B) Photomicrograph of hydrozincite forming on the surface of bacterial exopolysaccharide secretions.

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