Magnesium and zinc stable isotopes as a new tool to understand Mg and Zn sources in stream food webs

KAI NILS NITZSCHE,1,3,† KI-CHEOL SHIN,1 YOSHIKAZU KATO,1,4 HIROMITSU KAMAUCHI,1 SHOTARO TAKANO,2 AND ICHIRO TAYASU1

1Research Institute for Humanity and Nature (RIHN), 457-4 Motoyama, Kamigamo, Kita-ku, Kyoto 603-8047 Japan
2Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011 Japan

Citation: Nitzsche, K. N., K.-C. Shin, Y. Kato, H. Kamauchi, S. Takano, and I. Tayasu. 2020. Magnesium and zinc stable isotopes as a new tool to understand Mg and Zn sources in stream food webs. Ecosphere 11(8):e03197. 10.1002/ecs2.3197

Abstract. Non-traditional stable isotopes of metals were recently shown as new dietary tracers in terrestrial and marine mammals. Whether these metal stable isotopes can be used to understand feeding habits in stream food webs is not known yet. In this study, we explored the potential of stable isotopes of essential Mg (δ26Mg) and Zn (δ66Zn) as a new tool in stream ecology. For this purpose, we determined δ26Mg and δ66Zn values of stream organisms and their potential metal sources in upper and lower reaches of two streams in the Lake Biwa catchment, Central Japan. Our goals were (1) to explore variations in δ26Mg and δ66Zn across organisms of different feeding habits and (2) to understand Mg and Zn sources to stream organisms. Overall, δ26Mg and δ66Zn values of organisms were neither related to each other, nor to δ13C and δ15N values, indicating different elemental sources and factors controlling isotopic fractionation depending on element and taxa. Low δ26Mg values in filter-feeding caddisfly larvae and small gobies indicated aqueous Mg uptake. Higher δ26Mg values in leaf-shredding crane fly and grazing mayfly larvae suggested Mg isotopic fractionation during Mg uptake from the diet. While the δ26Mg values of stonofly nymphs reflected those of caddisfly larvae as a potential prey, the highest δ26Mg values found in dobsonfly nymphs can be explained by 26Mg enrichment during maturing. δ66Zn values of caddisfly and mayfly larvae indicated Zn was a mixture of aqueous and dietary available Zn, while higher δ66Zn values in crane fly larvae pointed to Zn isotopic fractionation during Zn uptake from plant litter. δ66Zn values in stonefly and dobsonfly nymphs were often in the range of those of caddisfly larvae as their prey, while dragonfly nymphs and small goby were depleted in 66Zn relative to their dietary Zn sources. We conclude that δ26Mg is a promising indicator to assess Mg sources in stream ecology depending on taxa, while the use of δ66Zn is limited due to the complexity in Zn sources.

Key words: aquatic macroinvertebrates; feeding habits; goby; magnesium; non-traditional stable isotopes; stream; zinc.

Received 13 April 2020; revised 27 April 2020; accepted 4 May 2020; final version received 1 June 2020. Corresponding Editor: Whitney S. Beck.

Copyright: © 2020 The Authors. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

†E-mail: kai.nitzsche@jamstec.go.jp
INTRODUCTION

Some metals such as Mg and Zn are essential to most forms of life including aquatic organisms (Maret 2016). These metals play an important metabolic role in the bodies of organisms and in particular in regulating aquatic insect homeostasis owing to their high enzymatic functions (Dow 2017). While Mg, a macronutrient, is involved in DNA and RNA structuring by binding with ATP, Zn, a micronutrient, is an important cofactor in many enzymes. In streams and rivers, Zn is present in the particulate (biotic and abiotic) and dissolved phases, both bioavailable forms to aquatic insects. As such, excess Zn concentrations in streams and rivers can have toxic effects leading to declines in diversity and richness of stream insect larvae (e.g., Clements et al. 2000, Cain et al. 2004). On the other hand, aquatic insects can take up metals from their diet; however, large uncertainties exist on the relative contributions from water vs. the diet, which depend on the feeding habits and on the metal.

Aquatic insects are assumed to take up and accumulate metals whether essential or not from the diet and from the water (Hare 1992, Goodyear and McNeill 1999, Rainbow 2007). To date, information on metal sources to specific taxa is derived both from field and from laboratory studies (Brix et al. 2011). The majority of these studies focused on the bioaccumulation of heavy metals with strong toxic effects such as Cd, Cu, Ni, Pb, and Zn, paying little attention to the source of macronutrients such as Mg (Goodyear and McNeill 1999, Brix et al. 2011). For instance, grazing and collector–gatherer mayfly nymphs are assumed to obtain heavy metals (Cd, Cu, Zn) from their diet (Cain et al. 2011, Kim et al. 2012, Poteat and Buchwalter 2014) with absorption possible in the midgut epithelium (Dow 1986, Huang et al. 2015). Less information exists on predators, but it is assumed that the prey is the main metal source (Hare et al. 2003, Martin et al. 2007). Consequently, relative metal contributions from water and the diet differ among taxa demonstrating the need for a better understanding of tracing metal sources. Traditionally, stream food webs are assessed via stable C and N isotope ratios and radiocarbon (Middelburg 2014, Ishikawa et al. 2016). While C isotope ratios ($\delta^{13}C$, $\Delta^{14}C$) provide insights into relative contributions of periphyton vs. plant litter as main dietary sources, N isotope ratio ($\delta^{15}N$) is used to assess trophic positions. Recent developments in mass spectrometry have allowed for measuring non-traditional isotopes of metals such as Mg ($\delta^{26}Mg$) and Zn ($\delta^{66}Zn$) that could be used to assess Mg and Zn sources and trophic transfer.

Using $\delta^{66}Zn$ values, Wanty et al. (2017) showed that approximately 85% Zn in grazing mayfly nymphs originated from diatom as diet. In a field study, similar $\delta^{66}Zn$ values in predatory stonefly nymphs and leaf-shredding caddisfly larvae in a Canadian creek indicated that similar Zn sources existed (Evans et al. 2016). Nevertheless, the number of feeding guilds was restricted in Evans et al. (2016) and larger differences in $\delta^{66}Zn$ may exist for aquatic insects that rely on other metal sources. For instance, freshwater diatoms were enriched in $\delta^{66}Zn$ by up to 0.2‰ compared to the growth medium (Gélabert et al. 2006) and lower $\delta^{66}Zn$ values were found in plant leaves relative to shoots and roots (Viers et al. 2007). These differences in $\delta^{66}Zn$ values across potential diets could be reflected in consumers. Using $\delta^{66}Zn$ values in bone and tooth material of African mammals, Jaouen et al. (2016a) were able to distinguish between browsers and grazers both having higher $\delta^{66}Zn$ values than plants. Owing to the depletion in $^{66}Zn$ relative to the global isotopic composition of the body (Balter et al. 2013), carnivores were found to have lower $\delta^{66}Zn$ values than herbivores (Jaouen et al. 2013, 2016a). Similarly, a decrease in $\delta^{66}Zn$ across the trophic chain was found in arctic marine mammals (Jaouen et al. 2016b). Furthermore, $\delta^{26}Mg$ data of ecological samples mainly are derived from African mammals. Based on the tooth enamel, Martin et al. (2015) observed an enrichment in $^{26}Mg$ up the trophic chain owing to the $^{26}Mg$ enrichment in muscle relative to bone, while feces became depleted in $^{26}Mg$. However, the mechanisms behind this Mg and Zn isotopic fractionation are poorly understood but could be related to Mg and Zn transport through enterocytes during intestinal absorption (Martin et al. 2015, Jaouen et al. 2016a). Similarly, Mg and Zn isotope fractionation during intestinal absorption from the diet could be suspected for aquatic insects. Recent evidence exists that potential differences in
\(\delta^{26}\text{Mg}\) values across sources are reflected in stream organisms from streams with contrasting geology (Nitzsche et al. 2019). These authors suggested stream water was the main Mg source to caddisfly larvae and small goby, while grazing mayfly, leaf-shredding crane fly, and predatory stonefly larvae rather relied on the diet as main Mg source. Nevertheless, Nitzsche et al. (2019) did not determine \(\delta^{26}\text{Mg}\) values of dietary sources crucial in understanding Mg sources and Mg isotopic fractionation.

In this study, we determined the \(\delta^{26}\text{Mg}\) and \(\delta^{66}\text{Zn}\) values of stream organisms and their potential metal sources in two streams in the Lake Biwa catchment, Japan. Upper and lower reaches were sampled to account for potential human impacts. Our goals were (1) to explore variations in \(\delta^{26}\text{Mg}\) and \(\delta^{66}\text{Zn}\) values across organisms of different feeding habits and (2) to understand Mg and Zn sources to stream organisms by considering isotopic discrimination. Furthermore, we aimed to link \(\delta^{26}\text{Mg}\) and \(\delta^{66}\text{Zn}\) values to traditional stable C and N isotope ratios to relate \(\delta^{26}\text{Mg}\) and \(\delta^{66}\text{Zn}\) values to primary food sources (periphyton vs. plant litter) and trophic transfer.

**Materials and Methods**

**Study site**

In May 2018 and November 2018, we sampled the upper and lower reaches of the Yasu River and the Ado River in the Lake Biwa catchment, Central Japan (Fig. 1). Upper reaches are pristine and dominated by forests, while residential areas and rice paddies are present in lower reaches. Riparian areas are typically dominated by cypress (Cupressaceae), beeches, and oaks (Fagaceae; Ishikawa et al. 2014). Chert, sandstone, and shale of the Jurassic Tanba Group are the typical lithology in upper reaches of the Ado River, while Jurassic mudstone and sandstone (59%) and Late Cretaceous granite (41%) dominate upper reaches of the Yasu River (Geological Survey of Japan 2015). These rocks occur as cobbles in stream beds. Pliocene and Pleistocene unconsolidated sediments of the Kobiwako Group and Quaternary sediments are present in catchments of lower reaches. Sedimentary rock cobbles from the Tanba Group remain present in stream beds of lower reaches with some sandy proportions in the Ado River stream bed.

**Physicochemical characteristics of stream water**

The water temperature, pH, and electric conductivity were measured in situ with probes (Laqua Conductivity Meter ES-71 and Laqua pH Meter D-71; Horiba Scientific, Piscataway, New Jersey, USA).

**Sampling**

Five L of stream water was taken and cooled before further preparation. Aquatic macroinvertebrates and small goby were collected with hand nets by washing cobbles. Terrestrial leaf litter from the most abundant species was collected from litter packs within streams. Fagaceae comprised of beeches and oaks was the most dominant family of identified plant litter. Plant litter was absent at lower reaches in May except for Quercus myrsinaefolia (oak). In November, plant litter in the upper site of the Ado River was largely dominated by Sorbus alnifolia (Korean mountain ash), while Euptelea polyandra (a Japanese endemic deciduous tree species) dominated in the upper site of the Yasu River. Periphytic algae attached to rock cobbles (hereafter periphyton) were removed from several submerged cobbles using a nylon brush and subsequently rinsing cobbles surfaces with distilled water (DW). The slurry was then transferred into 100-mL polypropylene bottles.

**Sample preparation**

Water samples were filtered using 0.20-µm cellulose acetate filters with vacuum filtration. A 100 mL subsample was taken, and the remaining filtered water was acidified. Terrestrial leaf litter was oven-dried at 65°C for 48 h after gently rinsing with DW to remove mineral particles. Leaf litter species were then identified, and three leaves of each species were ground with a multi-beads shucker (Yasui Kikai, Osaka, Japan). The periphyton slurry was three times decanted with DW to remove mineral particles and then freeze-dried. We examined dried periphyton samples under the binocular using up to 10 times magnification and removed visible mineral particles, small larvae of aquatic organisms, and plant residues. An overview of the total number of fish and aquatic macroinvertebrate samples with respect to stream name, location, and sampling month is given in Appendix S1: Table S1. Aquatic macroinvertebrates were classified based on...
species, genus, or family levels and categorized according to feeding habits based on the observations of Takemon (2005): grazer (GR), shredder (SH), filter feeder (FF), collector–gatherer (CG), omnivore (O), and predator (PR). Samples include larvae of caddisfly (Hydropsychidae spp., FF; Stenopsyche marmorata, FF; Rhyacophilidae, PR) and crane fly (Tipulidae, not further
classified, SH), and nymphs of mayfly (Baetis spp., GR; Heptageniidae spp., GR), stonefly (Kamimuria spp., PR; Oyama spp., PR), dragonfly (Gomphidae spp., PR), and dobsonfly (Protonemus grandis, PR), as well as small freshwater crab (Geothelphusa dehaani, CG) and shrimps (Atyiidae spp., O). Collected animals were previously kept in stream water in the laboratory for 24 h to purge their gut contents as far as possible prior to analysis. While Solà and Prat (2006) showed larvae of crane fly (S. marmorata), dobsonfly, and dragonfly, respectively, to obtain digestion. Periphyton samples were centrifuged at 2610 g for 10 min, and the supernatant was extracted. The remaining periphyton pellet was washed with DW and centrifuged again, and the supernatant was extracted and mixed with the supernatant from the first centrifugation step. This washing step was repeated one more time. The combined supernatants were then evaporated to full dryness and re-dissolved in 1-mol/L HNO₃ for further analysis.

**Chemical and isotopic analyses**

For carbon and nitrogen stable isotope analysis, solid subsamples of aquatic macroinvertebrates, fish muscles, periphyton, and plant litter were weighed into tin capsules combusted in an elemental analyzer (Flash 1112, Thermo Fisher Scientific, Bremen, Germany) at the Research Institute for Humanity and Nature (RIHN) in Kyoto, Japan. Isotope ratios were measured with an isotope ratio mass spectrometer (Delta V advantage, Thermo Fisher Scientific) that was coupled to the elemental analyzer via an interface (Conflo IV, Thermo Fisher Scientific). The isotopic values are expressed in delta notation (‰), relative to VPDB (Vienna Pee Dee Belemnite) for carbon and N₂ in air for nitrogen. Isotopic calibration was to CERKU-01 (DL-alanine), CERKU-02 (L-alanine), and CERKU-03 (glycine; Tayasu et al. 2011). Analysis of internal laboratory standards ensured that the estimates of the isotopic values were precise to within 0.04‰ for δ¹³C and 0.07‰ for δ¹⁵N.

Aliquots of all digested samples and of stream water were analyzed for elemental concentrations using a quadrupole inductively coupled plasma mass spectrometry (ICP-MS; 7500cx; Agilent Technologies, Tokyo, Japan) at the RIHN. Magnesium was purified from solutions containing up to 5 µg Mg using AG50W-X12 (200–400 mesh) resin with 1-M HNO₃ as the eluent based on a modified version of the protocol described in An et al. (2014). We ensured that almost no Mg fractionation occurred (recovery rate was typically >99%) during the purification by collecting 1 mL before and after the Mg elution peak and analyzing the Mg concentrations with the ICP-MS.

Purified samples were diluted to 2% HNO₃ and a concentration of 200 ppb. Magnesium isotope ratios were measured on these solutions under wet plasma conditions using a Neptune Plus
Multicollector ICP-MS (Thermo Scientific) at the RIHN. Samples were analyzed using the standard–sample–standard bracketing method with the Dead Sea Mg metal (DSM-3) standard. Specifically, three separate analyses of the same sample solution were conducted, for which uncertainties were reported as two standard deviations (2σ). The 26Mg/24Mg and 25Mg/24Mg ratios were expressed in delta notation (in ‰ units) relative to the DSM-3 following Eq. 1 with \( x = 25 \) or 26:

\[
\delta^{26}\text{Mg} = \left( \frac{^{26}\text{Mg} / ^{24}\text{Mg}}{^{26}\text{Mg} / ^{24}\text{Mg}}_{\text{DSM-3}} - 1 \right) \times 1000 \quad (1)
\]

The long-term repeated measurement of DSM-3 yielded a \( \delta^{26}\text{Mg} \) value of 0.00 ± 0.16‰ (\( n = 45 \); 2σ; over approximately 12 months). Furthermore, the accuracy of the measurements was assessed by analyzing the interlaboratory standard Cambridge-1 (CAM1), which underwent the same purification protocol as our samples. The repeated measurement of CAM1 yielded a \( \delta^{26}\text{Mg} \) value of −2.61 ± 0.18‰ (\( n = 32 \); 2σ), which was in agreement with published values elsewhere (e.g., Tippert et al. 2008, Bolou-Bi et al. 2012, Kimmig et al. 2018). When \( \delta^{26}\text{Mg} \) was plotted against \( \delta^{25}\text{Mg} \), all the analyzed samples in the study showed a line with a slope of 0.517 ± 0.001 (2σ; not shown). This slope was close to the theoretical equilibrium slope of 0.521 (Young and Galy 2004).

Given low Zn concentrations in stream water, we preconcentrated Zn and removed alkali metals and alkaline earth metals by chelating extraction (Takano et al. 2017) at the Institute for Chemical Research, Kyoto University, Japan. Briefly, we passed 300–1200 mL stream water containing 1.2 µg Zn through NOBIAS Chelate PA-1L columns. The resin was then rinsed with 75 mL of DW. Finally, we eluted Zn with 8–10 mL of 1 M HNO3.

Zinc was purified from solutions containing up to 2.5 µg Zn with anion-exchange resin AG1x8 (200–400 mesh) resin in Cl form using a modified version of the protocol described in Borrok et al. (2007). We used 6 mL of 8 mol/L HCl, 5 mL of 3 mol/L HCl, 4 mL of 0.4 mol/L HCl, and finally 2 mL of DW for the elution.

Zinc isotope ratios of purified samples were determined using the same sample and measurement conditions as for Mg (200 ppb Zn in 2% HNO3, wet plasma, standard–sample bracketing). Instrumental mass fractionation was corrected using Cu-doping (see Toutain et al. 2008) by adjusting Cu/Zn ratios to 1:1 in samples and using the newly developed AA-ETH standard, which has an offset of 0.28 ± 0.02‰ relative to the commonly used JMC-LYON standard (Archer et al. 2017). To allow for comparison with previously published literature, we express our \( \delta^{66}\text{Zn} / ^{64}\text{Zn} \) ratios in delta notation relative to the JMC-LYON standard following Eq. 2:

\[
\delta^{66}\text{Zn}_{\text{JMC-LYON}} = \left( \frac{^{66}\text{Zn} / ^{64}\text{Zn}}{^{66}\text{Zn} / ^{64}\text{Zn}}_{\text{AA-ETH}} - 1 \right) \times 1000 + 0.28. \quad (2)
\]

We refrained from error propagation associated with the conversion of \( \delta^{66}\text{Zn} \) of AA-ETH to \( \delta^{66}\text{Zn} \) of JMC-LYON as the analytical uncertainty was usually greater than the error related to the conversion. We ensured quality control of \( \delta^{66}\text{Zn} \) values by analyzing NIST 682 (high-purity zinc). The long-term repeated measurement of NIST 682 yielded a \( \delta^{66}\text{Zn} \) value of −2.40 ± 0.05‰ (\( n = 13 \), 2σ), which corresponds to values reported elsewhere (John et al. 2007, Conway et al. 2013). Furthermore, the accuracy of the measurements was assessed by analyzing the interlaboratory standard NIST 1566b (oyster tissue), which underwent the same purification protocol as our samples. The repeated measurement of NIST 1566b yielded a \( \delta^{66}\text{Zn} \) value of 0.71 ± 0.03‰ (\( n = 24 \), 2σ).

**Statistical analyses**

We used the Shapiro-Wilk test to test for normally distributed data and the Levene’s test to test for homoskedasticity. We performed Student’s t-test in order to compare \( \delta^{26}\text{Mg} \) and \( \delta^{66}\text{Zn} \) values in stream water with those in periphyton. In order to test for significant differences in stable isotope ratios in aquatic animals according to the stream name (Yasu vs. Ado), stream location (upper vs. lower), sampling month (November vs. May), and taxa and feeding habits, and their interaction effects, we performed multi-way analysis of variance (ANOVA). We performed Tukey’s HSD post hoc test to identify these differences. Overall, we found large variations in \( \delta^{26}\text{Mg} \) and \( \delta^{66}\text{Zn} \) values across different orders of predators (Figs. 2, 3). Therefore, we explored statistical differences with order rather than feeding habits as the explantory variable. Crane fly larvae and Japanese freshwater crabs...
(only present at upper reaches) and shrimps (only present at lower reaches) were not included in the statistical analysis. We refrained from using taxa and feeding habits in interaction terms due to the small number of animals. We used the Pearson product-moment correlation analyses to explore relationships between $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ with biomass, Mg and Zn concentration, and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. All statistical analyses were performed using R (version 3.6.3, R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/).

RESULTS

Concentrations and isotope ratios of Mg and Zn in metal sources

The physicochemical characteristics of stream water are summarized in Appendix S1: Table S2,

Fig. 2. $\delta^{26}\text{Mg} \pm 2\sigma$ values (in ‰) of metal sources and aquatic organisms of the four sampling locations. Filled symbols represent May 2018 data; open symbols represent November 2018 data.
while major elemental concentrations and isotopic compositions of Mg and Zn in the dissolved phase of stream water are reported in Appendix S1: Table S3. $\delta^{26}$Mg values of stream water ranged from $-0.95$‰ to $-0.60$‰, values that are comparable to those of streams and rivers that drain silicate rocks worldwide (e.g., Tippper et al. 2006, 2008, Wimpenny et al. 2011). $\delta^{66}$Zn values of stream water ranged from 0.18‰ to 0.33‰ and showed no differences between site and month. These $\delta^{66}$Zn values were similar with the average of the world rivers (0.33‰; Little et al. 2014).

Magnesium and zinc concentrations and isotope data of periphyton are reported in Appendix S1: Table S4. $\delta^{26}$Mg values in periphyton reflected those in stream water ($P = 0.30$; Fig. 2). On the other hand, $\delta^{66}$Zn values in
periphyton were significantly (on average 0.18‰) higher than those in stream water \((P = 0.008)\), but the offset was smaller for the Yasu River than for the Ado River (Fig. 3).

Magnesium and zinc concentrations and isotope data of leaf litter are reported in Appendix S1: Table S5. \(\delta^{26}\text{Mg}\) values ranged from −1.28 to −0.54‰, and \(\delta^{66}\text{Zn}\) values ranged from −0.62 to 0.25‰. Quercus salicina and Quercus myrsinaefolia usually had the lowest \(\delta^{26}\text{Mg}\) and \(\delta^{66}\text{Zn}\) values. However, Q. salicina and Q. myrsinaefolia are generally unfavorable to decomposers owing to their hardness and poor nutrition.

Concentrations and isotope ratios of Mg and Zn in consumers

Magnesium and zinc concentrations and \(\delta^{26}\text{Mg}\) and \(\delta^{66}\text{Zn}\) values of all analyzed stream organisms can be found in Appendix S1: Tables S6 and S7. Magnesium concentrations ranged from 0.54 to 3.78 mg/g. Mg concentrations were significantly higher in stream insects from the Ado River \((1.56 \pm 0.19 \, \text{mg/g}; \text{mean} \pm \text{SE}; \, n = 32)\) than from the Yasu River \((1.17 \pm 0.22 \, \text{mg/g}; \, n = 28; \, P = 0.007; \text{Table 1})\). We observed the lowest Mg concentrations in dragonfly nymphs \((0.88 \pm 0.11 \, \text{mg/g}, \, n = 6)\) and the highest Mg concentrations in stonefly nymphs \((2.06 \pm 0.24 \, \text{mg/g}, \, n = 13)\).

There were no significant differences in \(\delta^{26}\text{Mg}\) values of stream insects in May vs. November (Table 1). However, \(\delta^{26}\text{Mg}\) values of consumers were significantly higher in the upper site of the Ado River \((-0.36 \pm 0.10\%o, \, n = 17)\) than in the lower site of the Ado River \((-0.56 \pm 0.09\%o, \, n = 18)\) and than in the upper site of the Yasu River \((-0.59 \pm 0.07\%o, \, n = 15; \, P < 0.001; \text{Appendix S2 Fig. S1})\). We found \(\delta^{26}\text{Mg}\) values to increase in the following order: omnivore shrimps \((-1.36 \pm 0.07\%o, \, n = 3)\) < Japanese freshwater crab (collector-gatherer; −1.03 ± 0.03‰, \(n = 3)\) < demersal gobies \((-0.84 \pm 0.08\%o, \, n = 7)\) < filterer-feeding caddisflies (Hydropsychiidae spp., S. marmorata) and predatory caddisflies (Rhyacophilidae; −0.75 ± 0.03‰, \(n = 18)\) < predatory stoneflies (Kanimurina spp., Oyamia spp.; −0.67 ± 0.08‰, \(n = 13\) < leaf-shredding crane flies (Tipulidae; −0.54 ± 0.09‰, \(n = 3)\) < grazing mayflies (Baetis spp., Heptageniidae spp.; −0.37 ± 0.03‰, \(n = 16\) < predatory dragonflies (Gomphidae spp.; −0.21 ± 0.06‰, \(n = 6)\) < predatory dobsonflies (Protohermes grandis; 0.31 ± 0.09‰, \(n = 7)\).

Zinc concentrations of stream insects did not differ between the Ado River and the Yasu River (Table 2). In both streams, Zn concentrations were significantly higher in upper \((0.25 \pm 0.03 \, \text{mg/g}, \, n = 29)\) than in lower \((0.18 \pm 0.02 \, \text{mg/g}, \, n = 31; \, P = 0.020)\) reaches. Furthermore, mayflies had the highest Zn concentrations, which were significantly higher in the Yasu River \((0.38 \pm 0.03 \, \text{mg/g})\) than in the Ado River \((0.17 \pm 0.02 \, \text{mg/g})\). Dragonflies had the lowest Zn concentrations \((0.13 \pm 0.01 \, \text{mg/g})\).

There were no significant differences in \(\delta^{66}\text{Zn}\) values of stream insects between lower vs. upper

| Table 1. Results of the analysis of variance (ANOVA) for factors controlling \(\delta^{26}\text{Mg}\) and Mg concentrations of stream organisms. |
|----------------|----------------|-----|----------------|--------|--------|----------------|-----|--------|--------|--------|
| Explanatory variable | \(df\) | \(F\) | \(P\) | \(df\) | \(F\) | \(P\) |
| Stream | 1 | 3.3 | 0.078 | 1 | 9.0 | 0.005* |
| Location | 1 | 2.4 | 0.130 | 1 | 0.2 | 0.662 |
| Month | 1 | 3.2 | 0.084 | 1 | 0.0 | 0.980 |
| Order | 5 | 92.2 | <0.001* | 4† | 7.6 | <0.001* |
| Stream:Location | 1 | 20.6 | <0.001* | 1 | 0.0 | 0.875 |
| Stream:Month | 1 | 0.1 | 0.789 | 1 | 0.4 | 0.530 |
| Stream:Order | 5 | 1.3 | 0.289 | 4† | 1.6 | 0.185 |
| Location:Month | 1 | 0.6 | 0.445 | 1 | 0.4 | 0.541 |
| Location:Order | 5 | 4.0 | 0.005* | 4† | 0.5 | 0.729 |
| Month:Order | 5 | 2.7 | 0.034* | 4† | 1.1 | 0.366 |
| Stream:Location:Month | 1 | 0.0 | 0.942 | 1 | 0.4 | 0.512 |

† Mg concentration of fish muscles and bones was not included in the ANOVA.

*\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\).
Table 2. Results of the analysis of variance (ANOVA) for factors controlling $^{66}$Zn and Zn concentrations of stream organisms.

| Explanatory variable        | df | $F$       | $P$  | df | $F$       | $P$  |
|-----------------------------|----|-----------|------|----|-----------|------|
| Stream                      | 1  | 0.5       | 0.001* | 1  | 0.5       | 0.001* |
| Location                    | 1  | 0.5       | 0.001* | 1  | 0.5       | 0.001* |
| Month                       | 1  | 0.5       | 0.001* | 1  | 0.5       | 0.001* |
| Order                       | 4  | 0.5       | 0.46   | 4  | 0.5       | 0.46   |
| Stream:Location             | 1  | 0.5       | 0.001* | 1  | 0.5       | 0.001* |
| Stream:Month                | 1  | 0.5       | 0.001* | 1  | 0.5       | 0.001* |
| Location:Order              | 4  | 0.5       | 0.001* | 4  | 0.5       | 0.001* |
| Month:Order                 | 4  | 0.5       | 0.001* | 4  | 0.5       | 0.001* |

† Zn concentration of fish muscles and bones was not included in the ANOVA.
* $P < 0.05$, **$P < 0.001$.  

reaches and between May vs. November (Table 2; Appendix S2 Fig. S1). However, $^{66}$Zn values were significantly higher in the Ado River compared to the Yasu River (0.42 ± 0.03% vs. 0.20 ± 0.02%, respectively; $P < 0.001$). $^{66}$Zn values of caddisflies, mayflies, and stoneflies were often in a similar range. Among caddisflies, S. marmorata tended to have higher $^{66}$Zn values than Hydropsychidae spp. and Rhyacophilidae. Average $^{66}$Zn values of predatory aquatic insects decreased from stonefly (0.46 ± 0.04%) to dobsonfly (0.29 ± 0.03%) to dragonfly (0.05 ± 0.04%). $^{66}$Zn values of goby were on average 0.13 ± 0.04%.

$^{13}$C and $^{15}$N values and correlations with $^{26}$Mg and $^{66}$Zn values

We found $^{13}$C values in stream organisms were significantly lower in the Yasu River than in the Ado River (−22.4 ± 0.4% vs. −19.5 ± 0.3%, respectively; $P < 0.001$) and significantly lower in upper than in lower reaches (−22.3 ± 0.4% vs. −19.6 ± 0.3%, respectively; $P < 0.001$; Fig. 4 and Table 3). While $^{13}$C values were similar in the Ado River between May and November ($P = 0.423$), $^{13}$C values were higher in the Yasu River in November (−21.2 ± 0.5%) than in May (−23.4 ± 0.5%; $P = 0.004$). $^{13}$C values ranged from −28.0 ± 0.2% in crane fly larvae to −18.7 ± 1.8% in freshwater crabs.

$^{15}$N values of stream animals were significantly lower in upper (1.8 ± 0.3%) than in lower reaches (6.4 ± 0.3%; $P < 0.001$). While $^{15}$N values were similar between May (1.9 ± 0.5%) and November in upper reaches (1.6 ± 0.5%), $^{15}$N values were significantly higher in lower reaches in May (6.9 ± 0.4%) compared to November (5.8 ± 0.5%; $P < 0.001$). At upper reaches, average $^{15}$N values increased from primary consumers (grazer, filter feeder, shredder; 0.6 ± 0.5%) to secondary consumers (predators; 2.4 ± 0.5%) to fishes (3.7 ± 1.3%); and at lower reaches from primary consumers (5.5 ± 0.3%) to secondary consumers (predators; 6.4 ± 0.5%) to fishes (9.8 ± 0.6%).

We found no correlation between $^{26}$Mg and $^{66}$Zn values in consumers (Fig. 5). In order to relate $^{26}$Mg and $^{66}$Zn to primary food sources (periphyton vs. plant litter) and trophic transfer, we aimed for linking $^{26}$Mg and $^{66}$Zn values with $^{13}$C and $^{15}$N values, respectively. Overall, there were no significant correlations between $^{26}$Mg and $^{66}$Zn with $^{13}$C (Appendix S2 Figs. S2, S3). We only found a weak correlation between $^{26}$Mg and $^{15}$N for the lower site of the Ado River ($r = −0.46, P = 0.045$) and between $^{66}$Zn and $^{15}$N for the upper site of the Yasu River ($r = −0.65, P = 0.007$).

Variation in $^{26}$Mg and $^{66}$Zn values among Protohermes grandis and Kamimuria spp

In order to test for isotopic variation related to insect size and biomass, we determined $^{26}$Mg and $^{66}$Zn in single individuals of dobsonfly (Protohermes grandis) in May 2018 from the upper site of the Ado River, and of
Fig. 4. Scatter plots of δ¹⁵N against δ¹³C with respect to feeding habits of the four sampling locations. Filled symbols represent May 2018 data; open symbols represent November 2018 data.

Table 3. Results of the analysis of variance (ANOVA) for factors controlling δ¹³C and δ¹⁵N values of stream organisms.

| Explanatory variable       | df | F   | P     | df | F   | P     |
|----------------------------|----|-----|-------|----|-----|-------|
| Stream                     | 1  | 81.2| <0.001*| 1  | 0.2 | 0.638 |
| Location                   | 1  | 67.9| <0.001*| 1  | 1015.9| <0.001*|
| Month                      | 1  | 2.1 | 0.160 | 1  | 23.4 | <0.001*|
| Order                      | 5  | 6.1 | <0.001*| 5  | 50.3 | <0.001*|
| Stream:Location            | 1  | 0.2 | 0.675 | 1  | 308.4 | <0.001*|
| Stream:Month               | 1  | 14.0| <0.001*| 1  | 0.7 | 0.417 |
| Stream:Order               | 5  | 0.7 | 0.657 | 5  | 3.1 | 0.020*|
| Location:Month             | 1  | 0.4 | 0.523 | 1  | 9.0 | <0.001*|
| Location:Order             | 5  | 1.3 | 0.281 | 5  | 6.4 | <0.001*|
| Month:Order                | 5  | 1.1 | 0.375 | 5  | 2.9 | 0.027*|
| Stream:Location:Month      | 1  | 0.6 | 0.445 | 1  | 0.0 | 0.954 |

*P < 0.05, ***P < 0.001.
stonfly (*Kamimuria* spp.) in May 2018 from the upper site of the Yasu River, respectively (Appendix S1: Table S8). δ²⁶Mg values across Ado dobsonfly specimen displayed a range of 1.04‰, but only of 0.24‰ for δ⁶⁶Zn. δ²⁶Mg values in dobsonfly showed a positive correlation with biomass (*r* = 0.75, *P* = 0.030), but a negative correlation with Mg concentration (*r* = −0.88, *P* < 0.001; Fig. 6). No correlations between δ⁶⁶Zn with biomass and Zn concentration were found for dobsonfly. Overall, we found *Kamimuria* spp. specimen from the Yasu River displayed a range of 0.35‰ for δ²⁶Mg, but only of 0.17‰ for δ⁶⁶Zn. δ²⁶Mg in stonfly showed a positive correlation between biomass when the specimen with the lowest biomass is excluded from the correlation analysis (*r* = 0.90, *P* = 0.014) and with Mg concentration (*r* = 0.84, *P* = 0.021). With respect to stable C and N isotopes, only a significant correlation between δ²⁶Mg and δ¹³C values in *Kamimuria* spp. was observed (Appendix S2 Fig. S4).

**DISCUSSION**

Although differences in δ²⁶Mg and δ⁶⁶Zn values among taxa with different feeding habits were evident, these differences were not reliably predicted by feeding habits. δ²⁶Mg and δ⁶⁶Zn values in some taxa were in the range of those of metal sources. δ²⁶Mg and δ⁶⁶Zn values in other taxa showed an offset to higher or lower δ²⁶Mg and δ⁶⁶Zn values compared to those of metal sources indicating isotopic fractionation during Mg and Zn uptake. δ²⁶Mg and δ⁶⁶Zn values were neither related to each other, nor to δ¹³C and δ¹⁵N values pointing to different elemental sources and/or other mechanisms driving isotopic variation. In the following, we firstly discuss the potential use of δ²⁶Mg and δ⁶⁶Zn as
dietary indicators in stream ecology and similarities to mammals. Secondly, we discuss metal contributions from sources and isotopic fractionation effects across taxa of different feeding habits.

**Variability of isotopic ratios across feeding habits**

Ecologists use variations in stable isotope ratios across animals as dietary indicators and to estimate trophic positions. Traditionally, δ13C values allow for estimating relative contributions of main dietary sources (periphyton vs. plant litter), while δ15N values allow for assessing δ15N trophic positions in stream food webs (Middelburg 2014). We found δ13C values of primary consumers (grazers, shredders, filter feeders) were usually intermediate between those of periphyton and plant litter confirming previous results that aquatic insects are mostly generalists (Ishikawa et al. 2016; Fig. 4). Periphyton tended to have a relative higher contribution than plant litter in lower reaches as δ13C values of consumers were closer to δ13C values of periphyton, which agrees with the scarcity of plant litter at lower reaches. Furthermore, the overall higher δ15N values of predatory insects and fish compared to those of primary consumers agree well with the 15N trophic enrichment.

We found δ26Mg and δ66Zn values in consumers were neither related to each other (Fig. 5), nor to δ13C and δ15N values (Appendix S2 Figs. S2, S3), suggesting that δ26Mg and δ66Zn cannot be simply used to assess relative contributions of plant litter and periphyton as main Mg and Zn sources, and that δ26Mg and δ66Zn cannot be related to trophic level. Similarly, δ66Zn could not be related to δ13C and δ15N values in predatory stonefly nymphs and shredding caddisfly larvae in the Jackson Creek in Ontario, Canada (Evans et al. 2016). Thus, our data indicate (1) that different Mg, Zn, C, and N sources existed, (2) Mg and Zn isotopic fractionation during dietary Mg and Zn uptake irrespective of trophic position, (3) Mg and Zn isotopic fractionation related to insect growth and metamorphosis, and (4) variable rates of aqueous Zn uptake among taxa in relation to factors such as phylogeny and development (Buchwalter et al. 2008, Poteat and Buchwalter 2014, Cain et al. 2019) and to metal partitioning between aqueous and solid phases that could influence the relative contributions of aqueous and dietary metal uptake (Cain et al. 2011). These factors controlling δ26Mg and δ66Zn values in consumers will be discussed in detail in the following section as factors depend on taxa and feeding habits. We also need to keep in mind that seasonal variations in isotopic ratios of elemental sources could affect isotopic ratios in consumers. For instance, dietary C and N contributions can change over the year, and changes in agricultural contributions were shown to affect δ15N values in POM and periphyton (Karube et al. 2010, Ishikawa et al. 2014, 2016). Although the stream water and periphyton δ26Mg and δ66Zn values were similar between May and November, more frequent, for example, monthly variations in δ26Mg and δ66Zn values that we did not capture with our sampling design could exist. These variations in isotopic composition of sources could have impacted δ26Mg and δ66Zn values in organisms of different larval stages that may last from several weeks to a few years (Merrit et al. 2008).

The overall absence of significant differences in δ26Mg and δ66Zn values between upper vs. lower reaches (except for δ26Mg in the Ado River; Tables 1 and 2; Appendix S2 Fig. S1) indicates that land use (forest in upper reaches vs. residential areas and rice paddy fields in lower reaches) had only a little effect on Mg and Zn sources and/or indicates the overlap between isotope signatures of natural and anthropogenic sources. While δ26Mg of stream water is largely determined by the local geology (Brenot et al. 2008, Tipper et al. 2008, Nitzsche et al. 2019), larger impacts on δ66Zn of stream water and consumers could exist for more severe contamination such as from urban runoff and sewage from wastewater treatment plants (Chen et al. 2008, 2009).

Despite the poor relationships between δ26Mg and δ66Zn values with δ13C and δ15N values, we were able to distinguish some taxa of different feeding habits (except predatory caddisfly Rhyacophilidae and stonefly nymphs) based on δ26Mg values in aquatic insects (Fig. 2). As such, filter feeders that rely on fine particulate organic matter (FPOM) as their main diet had lower δ26Mg values than grazers and shredders that rely on periphyton and plant litter, respectively. The latter can in turn be distinguished from large predators (dobsonfly and dragonfly nymphs) that show the largest 26Mg enrichment (Fig. 2).
Fig. 6. Scatter plots of $\delta^{26}\text{Mg} \pm 2\sigma$ in dobsonflies (*Protohermes grandis*) of different sizes from Upper Ado in May 2018 against biomass (a) and against Mg concentration (c); of $\delta^{66}\text{Zn} \pm 2\sigma$ in dobsonflies against biomass (e)
Smaller predatory aquatic insects, that is, caddisflies and stoneflies, cannot be differentiated from filter feeders due to the similar range in δ²⁶Mg values. Collector–gatherers (Japanese freshwater crab) and omnivores (shrimp), both not aquatic insects, can be distinguished from other feeding guilds owing to their low δ²⁶Mg values. The ²⁶Mg enrichment in large predatory aquatic insects agrees with the ²⁶Mg enrichment across the trophic chain observed for African mammals (Martin et al. 2015). However, demersal goby, the highest predator in stream food webs, did not follow the trend of trophic enrichment in ²⁶Mg.

δ⁶⁶Zn patterns could not be generalized due to variations in δ⁶⁶Zn values of taxa across streams (Ado River vs. Yasu River), across locations (upper vs. lower), and given the small range in δ⁶⁶Zn values across taxa of different feeding habits within sampling locations. For instance, the similar δ⁶⁶Zn values between stoneflies and caddisflies agree with similar values in predatory stonefly nymphs and leaf-shredding caddisfly larvae in the Jackson Creek in Ontario, Canada (Evans et al. 2016), indicating similar Zn sources. We conclude that it is challenging to distinguish feeding habits based on δ⁶⁶Zn values although predatory fishes and dragonfly nymphs show an expected tendency to lower δ⁶⁶Zn values as observed for terrestrial and marine mammals (Jaouen et al. 2013, 2016a, b). Our data demonstrate the general importance of feeding habits on δ²⁶Mg values and partly on δ⁶⁶Zn values. In the following, we discuss possible Mg and Zn sources across taxa of each feeding habits to provide explanations for the lack of relationship between δ²⁶Mg and δ⁶⁶Zn values.

**Magnesium and zinc sources and isotopic fractionation depending on feeding habits**

Filter-feeding caddisflies (Hydropsychidae spp. *S. marmorata*) usually had similar or slightly elevated δ²⁶Mg values than stream water in agreement with our previous findings (Nitzsche et al. 2019). Again, we suggest the direct Mg uptake from the water via anal papillae as the main Mg source (Komnick 1977). On the other hand, intermediate δ⁶⁶Zn values in Hydropsychidae spp. between stream water and periphyton suggest Zn in Hydropsychidae spp. is a mixture of these two sources. The even higher δ⁶⁶Zn values in larger *S. marmorata* indicate Zn isotope fractionation during dietary Zn uptake from plant ingestion as part of fPOM. We hypothesize this Zn isotope fractionation to take place during intestinal absorption in the midguts (Huang et al. 2013) as suggested for African mammals (Jaouen et al. 2013, 2016a). Therefore, our results indicate that in contrast to Mg, dietary Zn acquisition plays an important role in filter-feeding caddisfly.

Shredders comprising of larvae of crane fly (Tipulidae) had higher δ²⁶Mg and δ⁶⁶Zn values than plant litter pointing to the preferential incorporation of metabolically useful ²⁶Mg and ⁶⁶Zn, respectively, during dietary Mg and Zn uptake in the midgut. Our results agree with herbivory African mammals that had higher δ²⁶Mg and δ⁶⁶Zn values in tooth enamel and bones than plants (Jaouen et al. 2013, 2016a, Martin et al. 2015).

Grazing mayflies (Baetis spp., Heptageniidae spp.) always had higher δ²⁶Mg values than periphyton indicating Mg isotope fractionation during intestinal Mg absorption from the diet, possible in the midguts of mayflies (Nowghani et al. 2017). On the other hand, intermediate δ⁶⁶Zn values between periphyton and the stream water suggest Zn contributions from these two sources. In fact, metal influx is possible via chloride cells mainly concentrated on the tracheal gills (Poteat and Buchwalter 2014, Nowghani et al. 2017) allowing for direct Zn uptake from stream water. We also have to keep in mind that mayflies can ingest detrital material (e.g., of plant origin) trapped in periphyton mats (Steinman 1996), which is in agreement with their
intermediate $\delta^{13}$C values. Thus, our data suggest that Zn in mayfly nymphs from natural streams is a complex mixture between dietary sources and stream water. This finding is in contrast to incubation studies of grazing mayfly Neocloeon triangulifer, for which periphyton accounted for the majority of Zn taken up (Kim et al. 2012, Wanty et al. 2017). Nevertheless, we acknowledge that Zn uptake pathways in mayfly may not simply be extrapolated to Baetis spp. and Heptageniidae spp. investigated in our study.

We found a wide range in $\delta^{26}$Mg and $\delta^{66}$Zn values across predators of different orders (caddisfly, stonefly, dragonfly, dobsonfly) emphasizing the complexity of the feeding habit predator. Despite being categorized as predator, the usually similar $\delta^{26}$Mg and $\delta^{66}$Zn values in Rhypocorhaphidae larvae and in stream water pointed to metal uptake through anal papillae similar to filter-feeding caddisflies. $\delta^{26}$Mg and $\delta^{66}$Zn values of stonefly nymphs (Kamimuria spp., Oyamia spp.) were often in the range of caddisfly larvae as potential prey, which agrees with our previous $\delta^{26}$Mg data (Nitzsche et al. 2019) and similar $\delta^{66}$Zn values between predatory stonefly nymphs and caddisfly larvae in a Canadian stream (Evans et al. 2016). To better understand potential mechanisms controlling $\delta^{26}$Mg and $\delta^{66}$Zn patterns among Kamimuria spp., several individuals different in biomass from the upper site of the Yasu River in May were analyzed. Our data indicate Mg isotopes fractionated during growth and/or Mg sources changed among individuals of different biomasses (indicative of age; Fig. 6b). As the $\delta^{26}$Mg values of individuals with lower biomass were in the range of $\delta^{26}$Mg stream water (except for one individual), we suggest a high contribution of aqueous Mg through chloride cells (Komnick 1977). On the other hand, the negative correlation between $\delta^{26}$Mg with $\delta^{13}$C values (Appendix S2 Fig. S4b) suggests a higher contribution of plant material to larger individuals leading to Mg isotope fractionation during intestinal Mg absorption from Mg-rich plant material. In contrast to $\delta^{26}$Mg, biomass did not control $\delta^{66}$Zn values highlighting the variability in $\delta^{66}$Zn values of sources to individuals of different ages although smaller individuals tended to reflect the $\delta^{66}$Zn values of stream water (Fig. 6 f,h). Consequently, $\delta^{26}$Mg and $\delta^{66}$Zn of Kamimuria spp. at the other sampling locations represent mixtures of individuals with varying aqueous and dietary Mg and Zn contributions.

We also analyzed several individuals of dobsonfly nymphs (Protohermes grandis) different in biomass from the upper site of the Ado River in May. We found that different dietary contributions did not explain $\delta^{26}$Mg and $\delta^{66}$Zn values given the lacking relationships with $\delta^{13}$C and $\delta^{15}$N values (Appendix S2 Fig. S4a,c). As even the smaller specimen showed an offset in $\delta^{26}$Mg relative to stream water (Fig. 6c), dobsonfly nymphs clearly fractionated Mg isotopes, possibly during intestinal Mg absorption from the diet in their midguts (Terra and Ferreira 2012). Furthermore, dobsonfly nymphs accumulated $\delta^{26}$Mg in their tissues during their up to 5 years of larval stages while excreting Mg (Fig. 6a, c). The molting of the exoskeleton, which happens between 10 and 12 times, could represent an explanation for the preferential loss of $\delta^{24}$Mg assuming that $\delta^{24}$Mg is accumulated in the molted skin. In contrast to $\delta^{26}$Mg, the lacking correlations between $\delta^{66}$Zn with biomass and with Zn concentration suggest the variability in $\delta^{66}$Zn of sources, which could also comprise leaf-shredding crane fly larvae enriched in $\delta^{66}$Zn. We conclude that dobsonfly larvae at other sampling locations than the upper site of the Ado River represent individuals of different ages driving $\delta^{26}$Mg enrichment, while $\delta^{66}$Zn values suggest the variability in $\delta^{66}$Zn of sources. Our data clearly show the difference between Mg and Zn sources and $\delta^{26}$Mg vs. $\delta^{66}$Zn fractionation among dobsonfly larvae.

Predatory dragonfly nymphs (Gomphidae spp.) tended to have higher $\delta^{26}$Mg and lower $\delta^{66}$Zn values than other aquatic insects (Figs. 2, 3). Again, we suggest Mg and Zn isotopic fractionation occurred during intestinal Mg and Zn absorption from the diet. Whether the Mg and Zn isotope fractionation is also related to aging has to be shown by future studies.

We also determined $\delta^{26}$Mg in bones and muscles, and $\delta^{66}$Zn in muscles of demersal goby (Cottus pollux, Rhinogobius kurodai) that have the highest trophic positions in the Yasu River and the Ado River (Ishikawa et al. 2014). In accordance with our previous study (Nitzsche et al. 2019), we found $\delta^{26}$Mg values in bones of goby to reflect those of stream water indicating that Mg was directly taken up from the water presumably via the gills (Flik and Verboon 1993). Our findings
agree well with $\delta^{26}\text{Mg}$ data from otolith of silver perch (*Bidyanus bidyanus*) in a laboratory study, which showed that more than 80% Mg was taken up from water (Woodcock et al. 2012). Furthermore, we now confirm Mg isotope fractionation between goby bones and muscle is negligible as we did not find significant differences in $\delta^{26}\text{Mg}$ values between muscle vs. bones (Appendix S2 Fig. S5). On the other hand, the sometimes lower $\delta^{66}\text{Zn}$ values in muscles than those in stream water implied Zn isotope fractionation during dietary Zn uptake. The importance of intestinal Zn uptake has previously been suggested for freshwater fishes (c.f. Bakke et al. 2010). Our results demonstrate different uptake pathways of Zn (via water and the diet) vs. Mg (primarily via the water) for freshwater fishes.

Collector-gatherers comprising of Japanese freshwater crab (*Geothelphusa dehaani*) had the lowest $\delta^{26}\text{Mg}$ values in their shells that were in the range of plant litter, indicating that freshwater crab relied on residues from decomposed plant litter as the main Mg source. This contradicts with $\delta^{13}\text{C}$ values of two specimens implying that freshwater crabs relied on periphyton rather than plant litter. Thus, it is possible that low $\delta^{26}\text{Mg}$ in shells of freshwater crab is a result of Mg isotope fractionation during intestinal Mg absorption and/or Mg isotope fractionation between shells made of chitin relative to soft muscles within their legs and bodies. Further research is required to identify the exact Mg isotope fractionation mechanisms.

Similar $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values between muscles and shells in omnivore shrimps (*Atyidae* spp.; Appendix S2 Fig. S6) suggest that Mg isotope fractionation between muscles and shells is negligible. Instead, the low $\delta^{26}\text{Mg}$ values point to an additional Mg source depleted in $^{26}\text{Mg}$ that we did not sample, for instance, detritus or macrophytes. On the other hand, the similar $\delta^{66}\text{Zn}$ values of shrimps with those of sources agree well with their omnivorous definition.

Future directions of non-traditional isotopes in stream ecology

By determining $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values alongside with Mg and Zn concentrations and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of stream organisms and their potential metal sources, our study highlighted differences in relative contributions of aqueous vs. dietary to taxa of different feeding habits. Our data indicate metal uptake from the diet during intestinal absorption induced Mg and to a lesser extent Zn isotopic fractionation as suggested for African mammals (Jaouen et al. 2013, 2016a, Martin et al. 2014, 2015). Thus, our data suggest similar mechanisms exist that drive Mg and Zn isotopic fractionation despite the physiological differences between aquatic insects and mammals. Nevertheless, experimental studies will have to further elucidate the physiology of Mg and Zn isotopic fractionation during metal uptake and loss by determining $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values of feces, abandoned exoskeletons, guts, and body fluids. These studies will have to be performed on different taxa of different ages. Furthermore, laboratory studies will have to test for changes in relative contributions of aqueous vs. dietary Mg and Zn by maintaining Mg and Zn concentrations in the water, which will be especially relevant to filter feeders. This study was limited to stable isotopes of essential Mg and Zn. Other essential metals such as Ca, Fe, or Cu or nonessential metals such as Cd and Pb exist whose sources could differ. Finally, we suggest non-traditional isotopes of metals can be used to trace direct metal sources (diet, water) allowing to trace the transfer of heavy metals through the trophic chain.

Acknowledgments

We thank C. Yoshimizu for her help with carbon and nitrogen stable isotope analysis, and M. Tsuchiya for her support on zinc preconcentration of stream water. We also thank Y. Saitoh for his remarks on sample processing, and S. Ueda, N. Yuzen, and A. Yasuda for their support in chemical analysis. This research was funded through the JSPS International Fellowship for Research in Japan via KAKENHI grant number 17F17755. The analytical work was partly supported by JSPS KAKENHI grant number 16H02524 and 18K19367 to I.T. We gratefully acknowledge the anonymous reviewers for their helpful comments and suggestions.

Literature Cited

An, Y., F. Wu, Y. Xiang, X. Nan, X. Yu, J. Yang, H. Yu, L. Xie, and F. Huang. 2014. High-precision Mg isotope analyses of low-Mg rocks by MC-ICP-MS. Chemical Geology 390:9–21.
Archer, C., et al. 2017. Inter-calibration of a proposed new primary reference standard AA-ETH Zn for zinc isotopic analysis. Journal of Analytical Atomic Spectrometry 32:415–419.

Bakke, A. M., C. Glover, and A. Krogdahl. 2010. Feeding, digestion and absorption of nutrients. Pages 57–110 in M. Grosell, A. P. Farrel and C. J. Brauner, editors. Fish physiology: the multifunctional gut of fish. Academic Press, Cambridge, Massachusetts, USA.

Balter, V., A. Lamboux, A. Zazzo, P. Telouk, Y. Leverrier, J. Marvel, A. P. Moloney, F. J. Monahan, O. Schmidt, and F. Albarède. 2013. Contrasting Cu, Fe, and Zn isotopic patterns in organs and body fluids of mice and sheep, with emphasis on cellular fractionation. Metallomics 5:1756–5901.

Bolou-Bi, E. B., N. Vigier, A. Poszwa, J. P. Boudot, and E. Dambrine. 2012. Effects of biogeochemical processes on magnesium isotope variations in a forested catchment in the Vosges Mountains (France). Geochimica Et Cosmochimica Acta 87:341–355.

Borrok, D. M., R. B. Wanty, W. I. Ridley, R. Wolf, P. J. Lamothe, and M. Adams. 2007. Separation of copper, iron, and zinc from complex aqueous solutions for isotopic measurement. Chemical Geology 242:400–414.

Brenot, A., C. Cloquet, N. Vigier, J. Carignan, and C. France-Lanord. 2008. Magnesium isotope systematics of the lithologically varied Moselle river basin, France. Geochimica Et Cosmochimica Acta 72:5070–5089.

Brix, K. V., D. K. DeForest, and W. J. Adams. 2011. The sensitivity of aquatic insects to divalent metals: a comparative analysis of laboratory and field data. Science of the Total Environment 409:4187–4197.

Buchwalter, D. B., D. J. Cain, C. A. Martin, L. Xie, S. N. Luoma, and T. Garland. 2008. Aquatic insect eco-physiological traits reveal phyllogenetically based differences in dissolved cadmium susceptibility. Proceedings of the National Academy of Sciences of the United States of America 105:8321–8326.

Cain, D. J., M. N. Croteau, and C. C. Fuller. 2019. Competitive interactions among H, Cu, and Zn ions moderate aqueous uptake of Cu and Zn by an aquatic insect. Environmental Pollution 255:113220.

Cain, D., M. N. Croteau, and S. Luoma. 2011. Bioaccumulation dynamics and exposure routes of Cd and Cu among species of aquatic mayflies. Environmental Toxicology and Chemistry 30:2532–2541.

Cain, D. J., S. N. Luoma, and W. G. Wallace. 2004. Linking metal bioaccumulation of aquatic insects to their distribution patterns in a mining-impacted river. Environmental Toxicology and Chemistry 23:1463–1473.

Chen, J., J. Bin, P. L. Gaillardet, and S. Huon. 2009. Zn isotopes in the suspended load of the Seine River, France: isotopic variations and source determination. Geochimica Et Cosmochimica Acta 73:4060–4076.

Chen, J., J. G. Bin, and P. Louvat. 2008. Zinc Isotopes in the Seine River Waters, France: a probe of anthropogenic contamination. Environmental Science and Technology 42:6494–6501.

Clements, W. H., D. M. Carlisle, J. M. Lazorchak, and P. C. Johnson. 2000. Heavy metals structure benthic communities in Colorado mountain streams. Ecological Applications 10:626–638.

Conway, T. M., A. D. Rosenberg, J. F. Adkins, and S. G. John. 2013. A new method for precise determination of iron, zinc and cadmium stable isotope ratios in seawater by double-spike mass spectrometry. Analytica Chimica Acta 793:44–52.

Dow, J. A. T. 1986. Insect midgut function. Pages 187–328 in P. D. Evans and V. B. Wigglesworth, editors. Advances in insect physiology. Academic Press, London, UK.

Dow, J. A. T. 2017. The essential roles of metal ions in insect homeostasis and physiology. Current Opinion in Insect Science 23:43–50.

Evans, R. D., W. Wang, H. E. Evans, and R. B. Georg. 2016. Variation in Zn, C, and N isotope ratios in three stream insects. Facets 1:205–216.

Flik, G., and P. M. Verboest. 1993. Calcium-transport in fish gills and intestine. Journal of Experimental Biology 184:17–29.

Gélabert, A., O. S. Pokrovsky, J. Viers, J. Schott, A. Boudou, and A. Feurtet-Mazel. 2006. Interaction between zinc and freshwater and marine diatom species: surface complexation and Zn isotope fractionation. Geochimica Et Cosmochimica Acta 70:839–857.

Geological Survey of Japan, AIST. 2015. Seamless Digital Geological Map of Japan (1:200,000). May 29, 2015 version. Geological Survey of Japan, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan.

Goodyear, K. L., and S. McNeill. 1999. Bioaccumulation of heavy metals by aquatic macro-invertebrates of different feeding guilds: a review. Science of the Total Environment 229:1–19.

Hare, L. 1992. Aquatic insects and trace metals: bioavailability, bioaccumulation, and toxicity. Critical Reviews in Toxicology 22:327–369.

Hare, L., A. Tessier, and U. Borgmann. 2003. Metal sources for freshwater invertebrates: pertinence for risk assessment. Human and Ecological Risk Assessment 9:779–793.

Huang, J. H., X. Jing, and A. E. Douglas. 2015. The multi-tasking gut epithelium of insects. Insect Biochemistry and Molecular Biology 67:15–20.
Ishikawa, N. F., Y. Kato, H. Togashi, M. Yoshimura, C. Yoshimizu, N. Okuda, and I. Tayasu. 2014. Stable nitrogen isotopic composition of amino acids reveals food web structure in stream ecosystems. Oecologia 175:911–922.

Ishikawa, N. F., H. Togashi, Y. Kato, M. Yoshimura, Y. Kohmatsu, C. Yoshimizu, N. O. Ogawa, N. Ohte, N. Tokuchi, N. Ohkouchi, and I. Tayasu. 2016. Terrestrial-aquatic linkage in stream food webs along a forest chronosequence: multi-isotopic evidence. Ecology 97:1146–1158.

Jaouen, K., M. Beasley, M. Schoeninger, J.-J. Hublin, and M. P. Richards. 2016a. Zinc isotope ratios of bones and teeth as new dietary indicators: results from a modern food web (Koobi Fora, Kenya). Scientific Reports 6:26281.

Jaouen, K., M. L. Pons, and V. Balter. 2013. Iron, copper and zinc isotopic fractionation up mammal trophic chains. Earth and Planetary Science Letters 374:164–172.

Jaouen, K., P. Szpak, and M. P. Richards. 2016b. Zinc isotope ratios as indicators of diet and trophic level in arctic marine mammals. PLOS ONE 11:1–13.

John, S. G., J. Genevieve Park, Z. Zhang, and E. A. Boyle. 2007. The isotopic composition of some common forms of anthropogenic zinc. Chemical Geology 245:61–69.

Karube, Z., Y. Sakai, T. Takeyama, N. Okuda, A. Kohzu, C. Yoshimizu, T. Nagata, and I. Tayasu. 2010. Carbon and nitrogen stable isotope ratios of macroinvertebrates in the littoral zone of Lake Biwa as indicators of anthropogenic activities in the watershed. Ecological Research 25:847–855.

Kim, K. S., D. H. Funk, and D. B. Buchwalter. 2012. Dietary (periphyton) and aqueous Zn bioaccumulation dynamics in the mayfly Centroptilum triangulifer. Ecotoxicology 21:2288–2296.

Kimmig, S. R., C. Holmden, and N. Bélanger. 2018. Biogeochemical cycling of Mg and its isotopes in a sugar maple forest in Québec. Geochimica Et Cosmochimica Acta 230:60–82.

Kornick, H. 1977. Chloride cells and chloride epithelia of aquatic insects. International Review of Cytology 49:285–329.

Little, S. H., D. Vance, C. Walker-Brown, and W. M. Landing. 2014. The oceanic mass balance of copper and zinc isotopes, investigated by analysis of their inputs, and outputs to ferromanganese oxide sediments. Geochimica Et Cosmochimica Acta 125:673–693.

Maret, W. 2016. The metals in the biological periodic system of the elements: concepts and conjectures. International Journal of Molecular Sciences 17:1–8.

Martin, C. A., S. N. Luoma, D. J. Cain, and D. B. Buchwalter. 2007. Cadmium ecophysiology in seven stonfly (Plecoptera) species: delineating sources and estimating susceptibility. Environmental Science & Technology 41:7171–7177.

Martin, J. E., D. Vance, and V. Balter. 2014. Natural variation of magnesium isotopes in mammal bones and teeth from two South African trophic chains. Geochimica Et Cosmochimica Acta 130:12–20.

Martin, J. E., D. Vance, and V. Balter. 2015. Magnesium stable isotope ecology using mammal tooth enamel. Proceedings of the National Academy of Sciences of the United States of America 112:430–435.

Merritt, R. W., K. W. Cummins, and M. B. Berg. 2008. An Introduction to the Aquatic Insects of North America. Fourth edition. Kendall/Hunt Publishing, Dubuque, Iowa, USA.

Middelburg, J. J. 2014. Stable isotopes dissect aquatic food webs from the top to the bottom. Biogeosciences 11:2357–2371.

Nitzsche, K. N., Y. Kato, K.-C. Shin, and I. Tayasu. 2019. Magnesium isotopes reveal bedrock impacts on stream organisms. Science of the Total Environment 688:243–252.

Nowghani, F., S. Jonusaite, T. Watson-Leung, A. Donini, and S. P. Kelly. 2017. Strategies of ionoregulation in the freshwater nymph of the mayfly Hexagenia rigida. Journal of Experimental Biology 220:3997–4006.

Poteat, M. D., and D. B. Buchwalter. 2014. Calcium uptake in aquatic insects: influences of phylogeny and metals (Cd and Zn). Journal of Experimental Biology 217:1180–1186.

Rainbow, P. S. 2007. Trace metal bioaccumulation: models, metabolic availability and toxicity. Environment International 33:576–582.

Solá, C., and N. Prat. 2006. Monitoring metal and metalloid bioaccumulation in Hydropsyche (Trichoptera, Hydropsychidae) to evaluate metal pollution in a mining river. Whole body versus tissue content. Science of the Total Environment 359:221–231.

Steinman, A. D. 1996. Effects of grazers on freshwater benthic algae. Pages 341–373 in R. J. Stevenson, M. L. Bothwell, and R. L. Lowe, editors. Algal ecology. Academic Press, Cambridge, Massachusetts, USA.

Takano, S., M. Tanimizu, T. Hirata, K.-C. Shin, Y. Fukami, K. Suzuki, and Y. Sohrin. 2017. A simple and rapid method for isotopic analysis of nickel, copper, and zinc in seawater using chelating extraction and anion exchange. Analytica Chimica Acta 967:1–11.

Takemon, Y. 2005. Life-type concept and functional feeding groups of benthos communities as indicators of lotic ecosystem conditions. Japanese Journal of Ecology 55:189–197.
Tayasu, I., R. Hirasawa, N. O. Ogawa, N. Ohkouchi, and K. Yamada. 2011. New organic reference materials for carbon- and nitrogen-stable isotope ratio measurements provided by Center for Ecological Research, Kyoto University, and Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology. Limnology 12:261–266.

Terra, W. R., and C. Ferreira. 2012. Biochemistry and molecular biology of digestion. Pages 365–418 in L. I. Gilbert, editor. Insect molecular biology and biochemistry. Academic Press, Cambridge, Massachusetts, USA.

Tipper, E. T., A. Galy, and M. J. Bickle. 2008. Calcium and magnesium isotope systematics in rivers draining the Himalaya-Tibetan-Plateau region: Lithological or fractionation control? Geochimica Et Cosmochimica Acta 72:1057–1075.

Tipper, E. T., A. Galy, J. Gaillardet, M. J. Bickle, H. Elderfield, and E. A. Carder. 2006. The magnesium isotope budget of the modern ocean: constraints from riverine magnesium isotope ratios. Earth and Planetary Science Letters 250:241–253.

Toutain, J. P., J. Sonke, M. Munoz, A. Nonell, M. Polvé, J. Viers, R. Freydier, F. Sortino, J. L. Joron, and S. Sumarti. 2008. Evidence for Zn isotopic fractionation at Merapi volcano. Chemical Geology 253:74–82.

Viers, J., P. Oliva, A. Nonell, A. Gélabert, J. E. Sonke, R. Freydier, R. Gainville, and B. Dupré. 2007. Evidence of Zn isotopic fractionation in a soil-plant system of a pristine tropical watershed (Nsimi, Cameroon). Chemical Geology 239:124–137.

Wanty, R. B., L. S. Balistrieri, J. S. Wesner, D. M. Walters, T. S. Schmidt, C. A. Stricker, J. M. Kraus, and R. E. Wolf. 2017. In vivo isotopic fractionation of zinc and biodynamic modeling yield insights into detoxification mechanisms in the mayfly Neocloeon triangulifer. Science of the Total Environment 609:1219–1229.

Wimpenny, J., K. W. Burton, R. H. James, A. Gannoun, F. Mokadem, and S. R. Gislason. 2011. The behaviour of magnesium and its isotopes during glacial weathering in an ancient shield terrain in West Greenland. Earth and Planetary Science Letters 304:260–269.

Woodcock, S. H., A. R. Munro, D. A. Crook, and B. M. Gillanders. 2012. Incorporation of magnesium into fish otoliths: determining contribution from water and diet. Geochimica Et Cosmochimica Acta 94:12–21.

Young, E. D., and A. Galy. 2004. The isotope geochemistry and cosmochemistry of magnesium. Reviews in Mineralogy and Geochemistry 55:197–230.

**Supporting Information**

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.3197/full