

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

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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 ($\delta^{26}\text{Mg}$) and Zn ($\delta^{66}\text{Zn}$) as a new tool in stream ecology. For this purpose, we determined $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ 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 $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ across organisms of different feeding habits and (2) to understand Mg and Zn sources to stream organisms. Overall, $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values of organisms were neither related to each other, nor to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, indicating different elemental sources and factors controlling isotopic fractionation depending on element and taxa. Low $\delta^{26}\text{Mg}$ values in filter-feeding caddisfly larvae and small gobies indicated aqueous Mg uptake. Higher $\delta^{26}\text{Mg}$ values in leaf-shredding crane fly and grazing mayfly larvae suggested Mg isotopic fractionation during Mg uptake from the diet. While the $\delta^{26}\text{Mg}$ values of stonefly nymphs reflected those of caddisfly larvae as a potential prey, the highest $\delta^{26}\text{Mg}$ values found in dobsonfly nymphs can be explained by ^{26}Mg enrichment during maturing. $\delta^{66}\text{Zn}$ values of caddisfly and mayfly larvae indicated Zn was a mixture of aqueous and dietary available Zn, while higher $\delta^{66}\text{Zn}$ values in crane fly larvae pointed to Zn isotopic fractionation during Zn uptake from plant litter. $\delta^{66}\text{Zn}$ 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 ^{66}Zn relative to their dietary Zn sources. We conclude that $\delta^{26}\text{Mg}$ is a promising indicator to assess Mg sources in stream ecology depending on taxa, while the use of $\delta^{66}\text{Zn}$ is limited due to the complexity in Zn sources.

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

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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 ($\delta^{13}\text{C}$, $\Delta^{14}\text{C}$) provide insights into relative

contributions of periphyton vs. plant litter as main dietary sources, N isotope ratio ($\delta^{15}\text{N}$) is used to assess trophic positions. Recent developments in mass spectrometry have allowed for measuring non-traditional isotopes of metals such as Mg ($\delta^{26}\text{Mg}$) and Zn ($\delta^{66}\text{Zn}$) that could be used to assess Mg and Zn sources and trophic transfer.

Using $\delta^{66}\text{Zn}$ values, Wanty et al. (2017) showed that approximately 85% Zn in grazing mayfly nymphs originated from diatom as diet. In a field study, similar $\delta^{66}\text{Zn}$ 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 $\delta^{66}\text{Zn}$ may exist for aquatic insects that rely on other metal sources. For instance, freshwater diatoms were enriched in ^{66}Zn by up to 0.2‰ compared to the growth medium (Gélabert et al. 2006) and lower $\delta^{66}\text{Zn}$ values were found in plant leaves relative to shoots and roots (Viers et al. 2007). These differences in $\delta^{66}\text{Zn}$ values across potential diets could be reflected in consumers. Using $\delta^{66}\text{Zn}$ values in bone and tooth material of African mammals, Jaouen et al. (2016a) were able to distinguish between browsers and grazers both having higher $\delta^{66}\text{Zn}$ 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 $\delta^{66}\text{Zn}$ values than herbivores (Jaouen et al. 2013, 2016a). Similarly, a decrease in $\delta^{66}\text{Zn}$ across the trophic chain was found in arctic marine mammals (Jaouen et al. 2016b). Furthermore, $\delta^{26}\text{Mg}$ 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 paddy fields 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 cobble 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 shocker (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

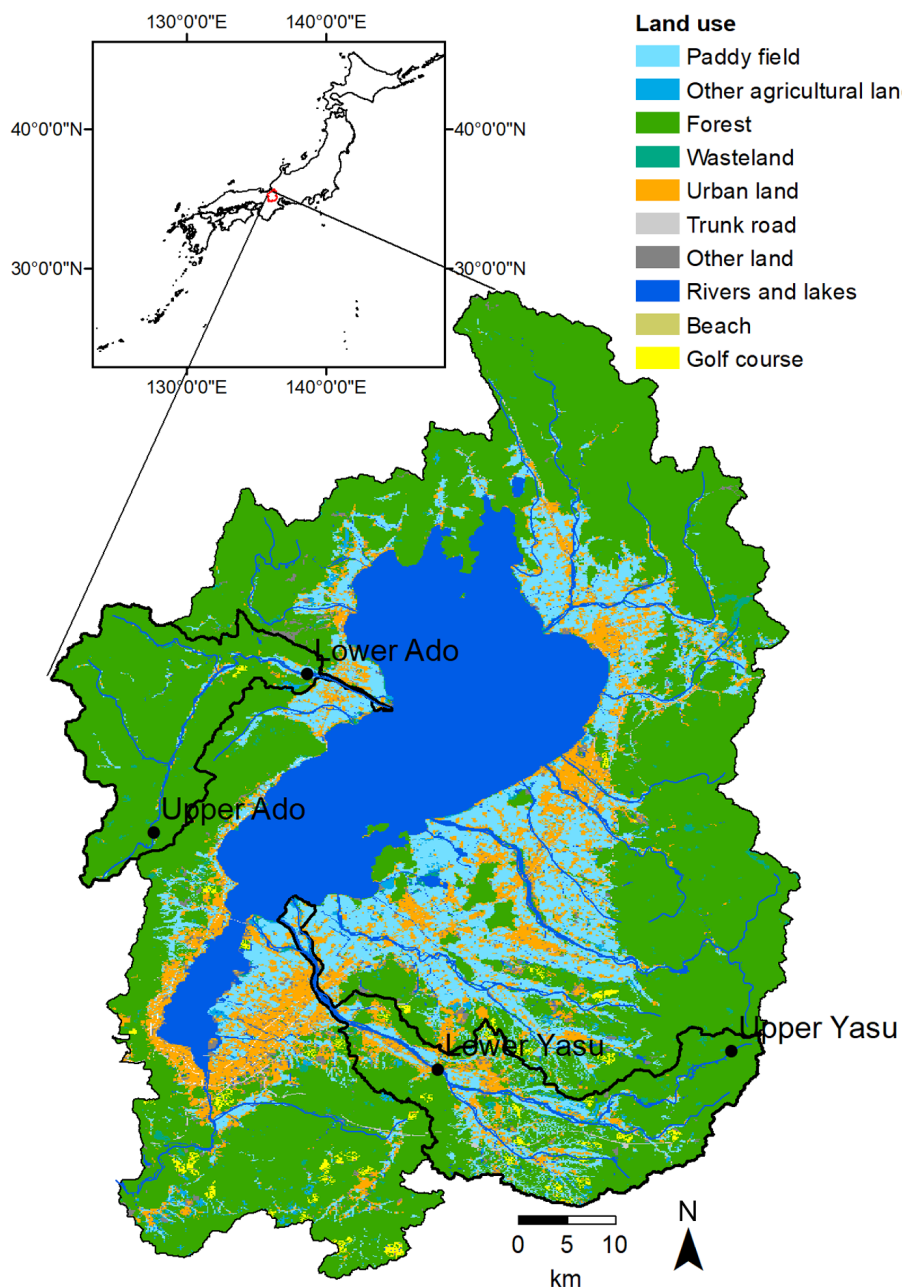


Fig. 1. Land-use map of the Lake Biwa catchment with sampling locations, watersheds of the Ado River and the Yasu River indicated by black bold lines, and location of the Lake Biwa catchment in Japan. Land-use data obtained from Land Use Fragmented Mesh Version 2.5.1 from the National Land Numerical Information, created by Ministry of Land, Infrastructure, Transport and Tourism, the Government of Japan.

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; *Oyamia* spp., PR), dragonfly (Gomphidae spp., PR), and dobsonfly (*Protohermes grandis*, PR), as well as small freshwater crab (*Geothelphusa dehaani*, CG) and shrimps (Atyidae 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 Hydropsychidae cleared its gut within 24 h, this time might be insufficient for other taxa. Thus, larvae of crane fly, large caddisfly (*S. marmorata*), dobsonfly, and dragonfly were additionally dissected to remove gut contents that could still be present. We separated shells from freshwater crabs and shrimps, which were gently rinsed by DW to remove attached muscles after obtaining muscle samples for shrimps. We obtained bones and muscles of goby (*Cottus pollux*, PR; *Rhinogobius kurodai*, PR/CG) and one chub (*Rhynchocypris oxycephalus jouyi*, PR) by dissection. Bones were additionally boiled at 80°C for 1 h to facilitate the removal of muscles that were still attached. All animal samples were freeze-dried for 48 h and then weighed to obtain dry weight (dw). We typically pooled several individuals of mayflies, stoneflies, and caddisflies, respectively, to obtain 2–100 mg dw for further analysis. For crane fly, dobsonfly, and dragonfly, analysis was conducted on single individuals. Several individuals different in size of stoneflies (*Kamimuria* spp.) from the upper site of the Yasu River and dobsonflies from the upper site of the Ado River in May were additionally prepared to test for isotopic fractionation related to body mass and therefore age. Animal samples were ground into a powder using pestle and mortar, and a subsample was taken for C and N stable isotope analysis. Ground animal and plant litter samples were digested with concentrated HNO₃ at 140°C for 72 h. H₂O₂ was added to remove organic matter still present in animal and plant litter samples. Several drops of concentrated hydrofluoric acid were added to plant litter samples to digest phytates containing silica. Samples were then evaporated to full dryness and re-dissolved in 1-M HNO₃ for further analysis. Periphyton samples were digested in 10% H₂O₂ for 48 h to avoid leaching of metals from mineral particles, which is possible to occur when using HNO₃ for

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 $^{26}\text{Mg}/^{24}\text{Mg}$ and $^{25}\text{Mg}/^{24}\text{Mg}$ ratios were expressed in delta notation (in ‰ units) relative to the DSM-3 following Eq. 1 with $x = 25$ or 26:

$$\delta^x\text{Mg} = \left(\frac{(^x\text{Mg}/^{24}\text{Mg})_{\text{sample}}}{(^x\text{Mg}/^{24}\text{Mg})_{\text{DSM-3}}} - 1 \right) \times 1000 \quad (1)$$

The long-term repeated measurement of DSM-3 yielded a $\delta^{26}\text{Mg}$ value of $0.00 \pm 0.16\text{‰}$ ($n = 45$; 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 $-2.61 \pm 0.18\text{‰}$ ($n = 32$; 2σ), which was in agreement with published values elsewhere (e.g., Tipper 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 mol/L HNO_3 .

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% HNO_3 , 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 $0.28 \pm 0.02\text{‰}$ relative to the commonly used JMC-LYON standard (Archer et al. 2017). To allow for comparison with previously published literature, we express our $^{66}\text{Zn}/^{64}\text{Zn}$ ratios in delta notation relative to the JMC-LYON standard following Eq. 2:

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

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 $\delta^{66}\text{Zn}$ of $-2.40 \pm 0.05\text{‰}$ ($n = 13$, 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 $0.71 \pm 0.03\text{‰}$ ($n = 24$, 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), 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 explanatory 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,

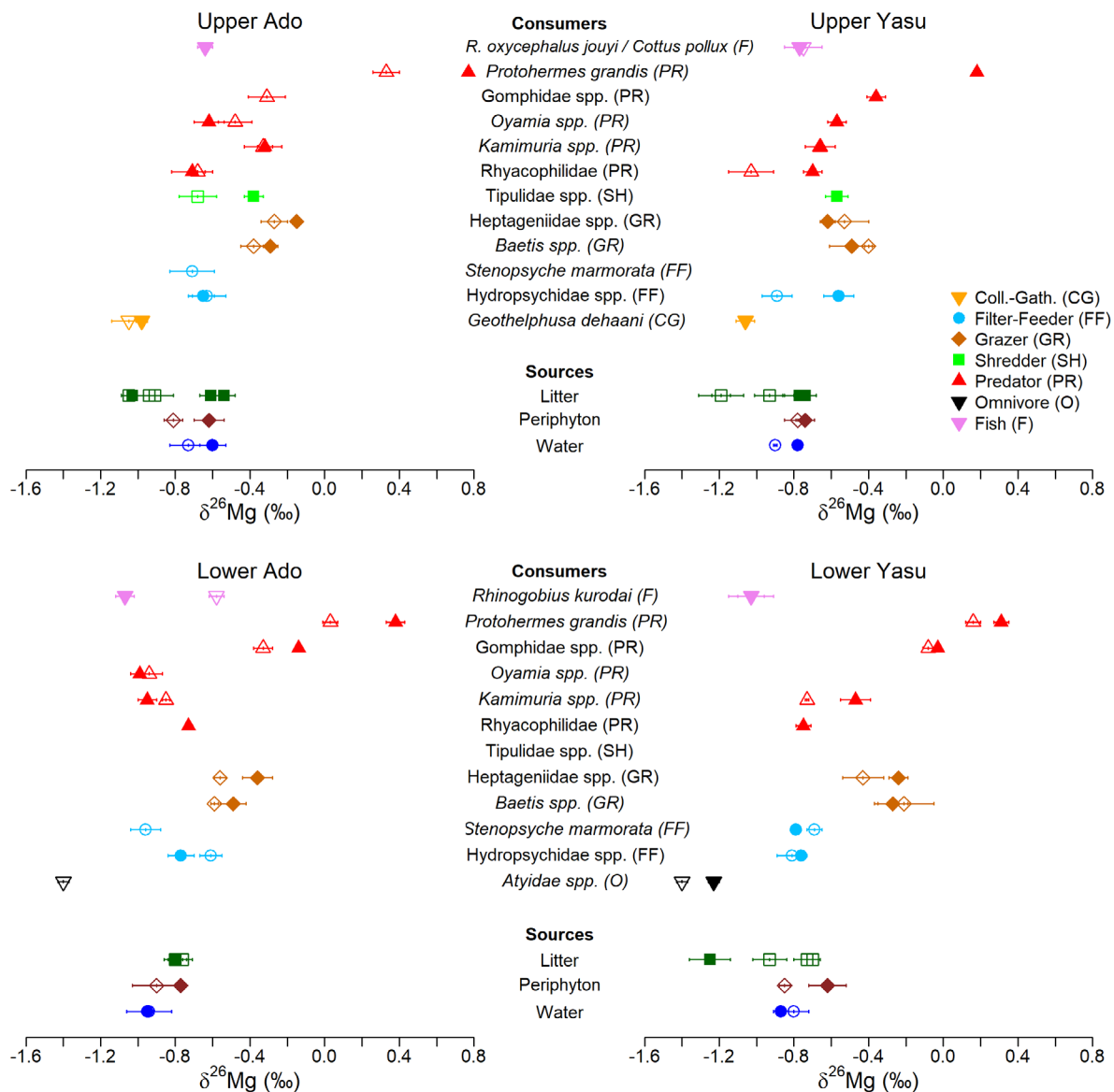


Fig. 2. $\delta^{26}\text{Mg} \pm 2\sigma$ values (in ‰) of metal sources and aquatic organisms of the four sampling locations. Filled symbols represent May 2018 data; open symbols represent November 2018 data.

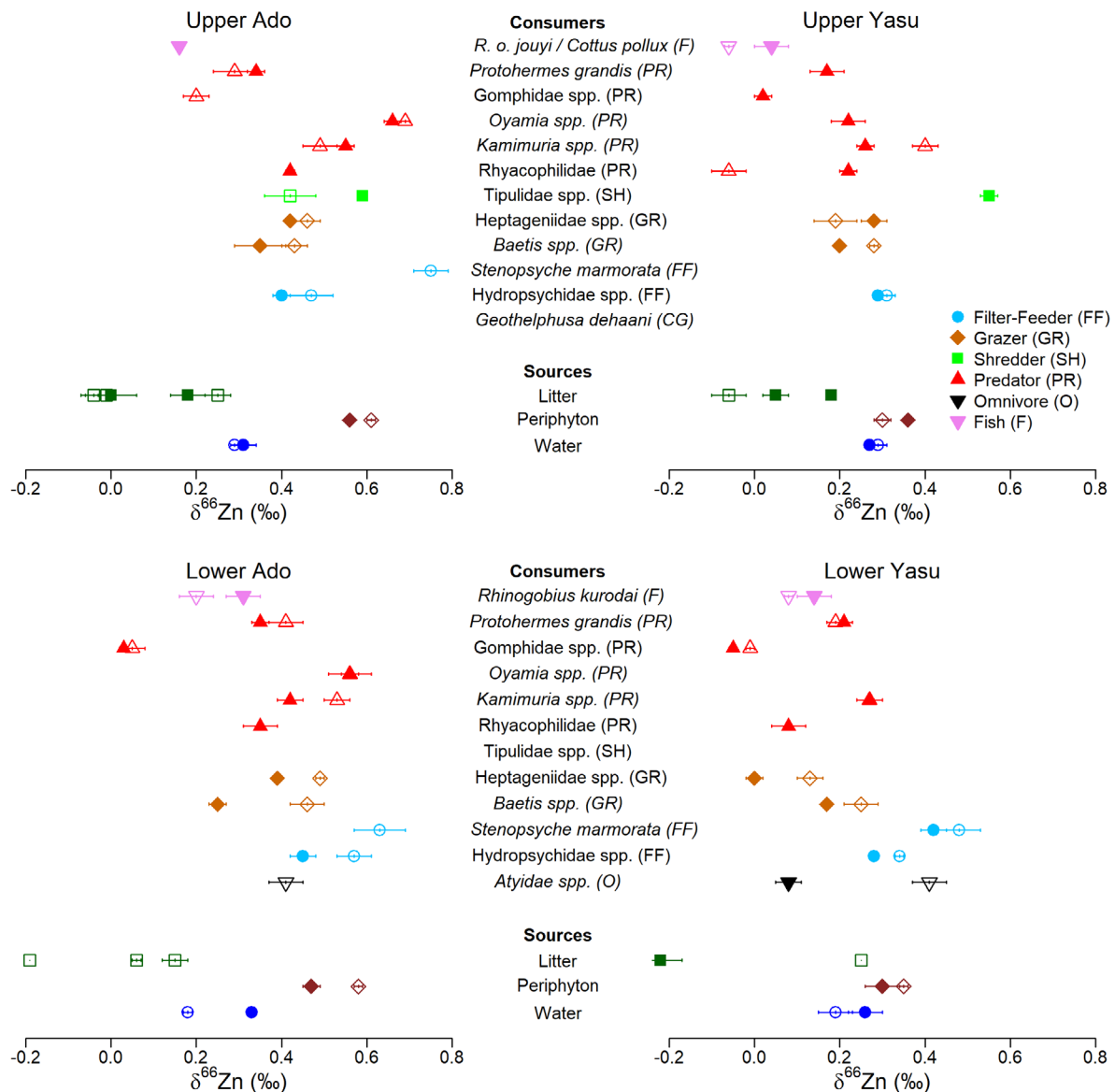


Fig. 3. $\delta^{66}\text{Zn} \pm 2\sigma$ values (in ‰) of metal sources and aquatic organisms of the four sampling locations. Filled symbols represent May 2018 data; open symbols represent November 2018 data.

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. $\delta^{26}\text{Mg}$ 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., Tipper et al. 2006, 2008, Wimpenny et al. 2011). $\delta^{66}\text{Zn}$ values of stream water ranged from 0.18‰

to 0.33‰ and showed no differences between site and month. These $\delta^{66}\text{Zn}$ 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. $\delta^{26}\text{Mg}$ values in periphyton reflected those in stream water ($P = 0.30$; Fig. 2). On the other hand, $\delta^{66}\text{Zn}$ 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 ± 0.19 mg/g; mean \pm SE; $n = 32$) than from the Yasu River (1.17 ± 0.22 mg/g, $n = 28$; $P = 0.007$; Table 1). We observed the lowest Mg concentrations in dragonfly nymphs (0.88 ± 0.11 mg/g, $n = 6$) and the highest Mg concentrations in stonefly nymphs (2.06 ± 0.24 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 ± 0.10 ‰, $n = 17$) than in the lower site of the Ado River (-0.56 ± 0.09 ‰, $n = 18$) and than in the upper site of the Yasu River (-0.59 ± 0.07 ‰, $n = 15$; $P < 0.001$; Appendix S2 Fig. S1). We found $\delta^{26}\text{Mg}$ values to increase in the following order: omnivore shrimps (-1.36 ± 0.07 ‰, $n = 3$) < Japanese freshwater crab (collector-gatherer; -1.03 ± 0.03 ‰, $n = 3$) < demersal goby (-0.84 ± 0.08 ‰, $n = 7$) < filterer-feeding caddisflies (Hydropsychidae spp., *S. marmorata*) and predatory caddisflies (Rhyacophilidae; -0.75 ± 0.03 ‰, $n = 18$) < predatory stoneflies (*Kamimuria* 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 (*Prothermes 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 ± 0.03 mg/g, $n = 29$) than in lower (0.18 ± 0.02 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 ± 0.03 mg/g) than in the Ado River (0.17 ± 0.02 mg/g). Dragonflies had the lowest Zn concentrations (0.13 ± 0.01 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	$\delta^{26}\text{Mg}$			Mg		
	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 $\delta^{66}\text{Zn}$ and Zn concentrations of stream organisms.

Explanatory variable	$\delta^{66}\text{Zn}$			Zn		
	df	F	P	df	F	P
Stream	1	95.4	<0.001*	1	2.7	0.110
Location	1	0.2	0.629	1	5.9	0.020*
Month	1	3.4	0.074	1	0.1	0.818
Order	5	21.1	<0.001*	4†	3.0	0.033*
Stream:Location	1	0.4	0.531	1	0.8	0.852
Stream:Month	1	1.3	0.254	1	0.0	0.931
Stream:Order	5	0.9	0.497	4†	3.8	0.011*
Location:Month	1	2.6	0.115	1	1.0	0.322
Location:Order	5	2.4	0.058	4†	0.9	0.494
Month:Order	5	1.1	0.359	4†	0.5	0.765
Stream:Location:Month	1	0.0	0.964	1	0.0	0.900

† 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, $\delta^{66}\text{Zn}$ values were significantly higher in the Ado River compared to the Yasu River ($0.42 \pm 0.03\text{‰}$ vs. $0.20 \pm 0.02\text{‰}$, respectively; $P < 0.001$). $\delta^{66}\text{Zn}$ values of caddisflies, mayflies, and stoneflies were often in a similar range. Among caddisflies, *S. marmorata* tended to have higher $\delta^{66}\text{Zn}$ values than Hydropsychidae spp. and Rhyacophilidae. Average $\delta^{66}\text{Zn}$ values of predatory aquatic insects decreased from stonefly ($0.46 \pm 0.04\text{‰}$) to dobsonfly ($0.29 \pm 0.03\text{‰}$) to dragonfly ($0.05 \pm 0.04\text{‰}$). $\delta^{66}\text{Zn}$ values of goby were on average $0.13 \pm 0.04\text{‰}$.

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

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

$\delta^{15}\text{N}$ values of stream animals were significantly lower in upper ($1.8 \pm 0.3\text{‰}$) than in lower reaches ($6.4 \pm 0.3\text{‰}$; $P < 0.001$). While $\delta^{15}\text{N}$

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

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

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

In order to test for isotopic variation related to insect size and biomass, we determined $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ in single individuals of dobsonfly (*Protohermes grandis*) in May 2018 from the upper site of the Ado River, and of

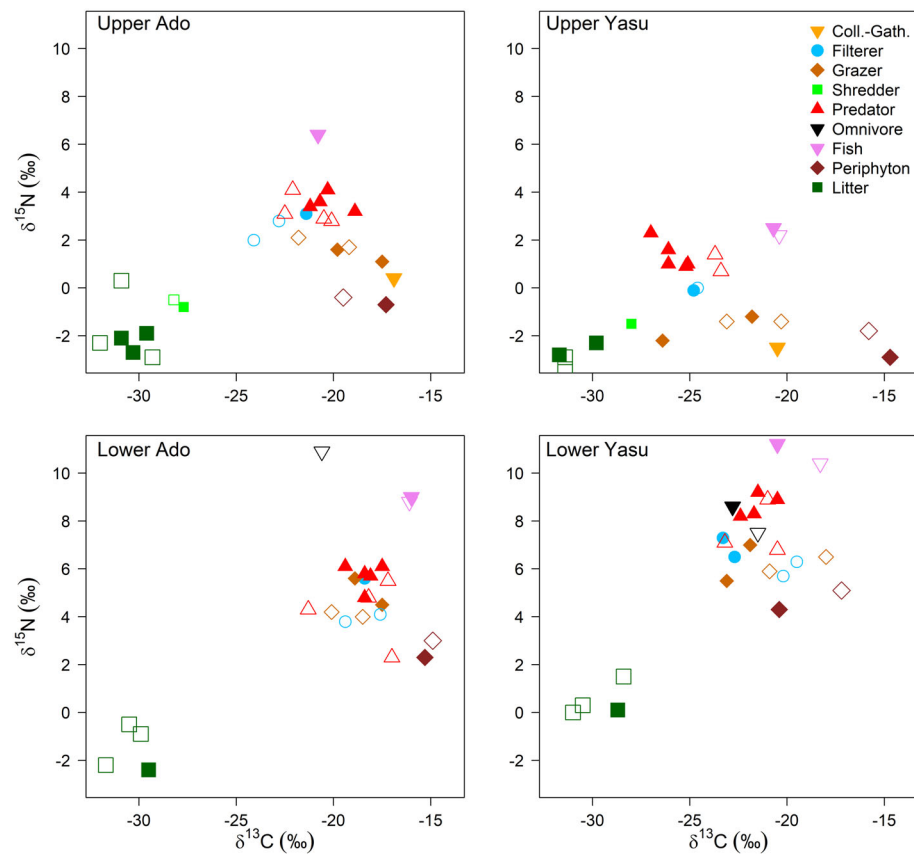


Fig. 4. Scatter plots of $\delta^{15}\text{N}$ against $\delta^{13}\text{C}$ with respect to feeding habits of the four sampling locations. Filled symbols represent May 2018 data; open symbols represent November 2018 data.

Table 3. Results of the analysis of variance (ANOVA) for factors controlling $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of stream organisms.

Explanatory variable	$\delta^{13}\text{C}$			$\delta^{15}\text{N}$		
	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

* $P < 0.05$, *** $P < 0.001$.

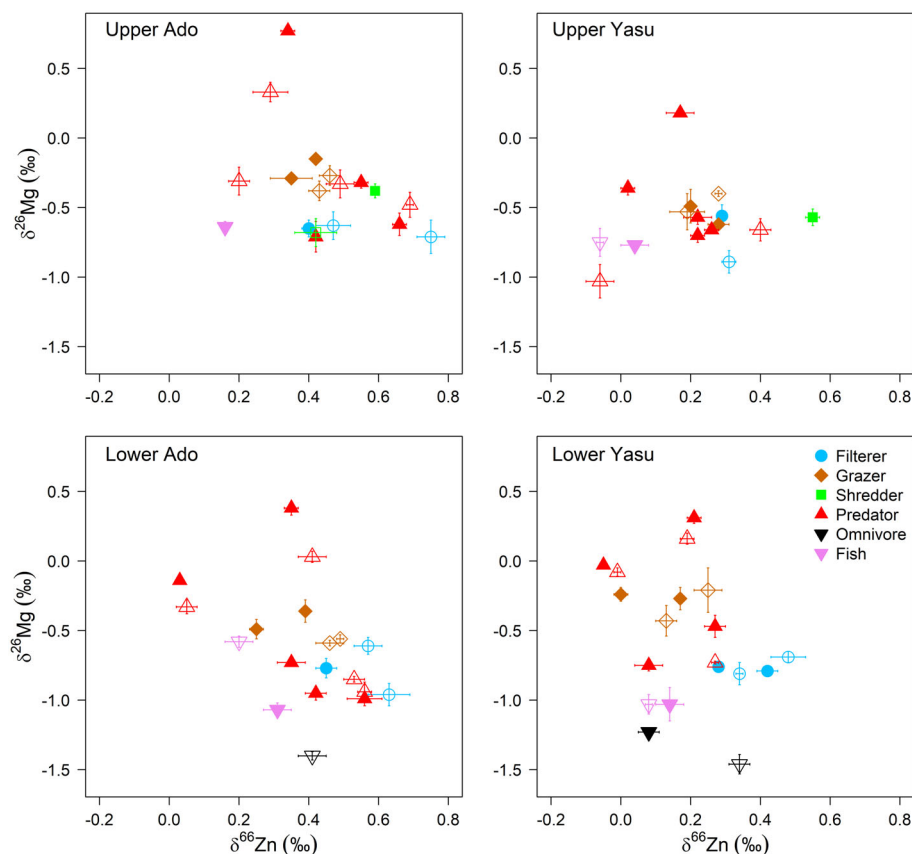


Fig. 5. Scatter plots of $\delta^{26}\text{Mg} \pm 2\sigma$ against $\delta^{66}\text{Zn} \pm 2\sigma$ with respect to feeding habits of the four sampling locations. Filled symbols represent May 2018 data; open symbols represent November 2018 data.

stonefly (*Kamimuria* spp.) in May 2018 from the upper site of the Yasu River, respectively (Appendix S1: Table S8). $\delta^{26}\text{Mg}$ values across Ado dobsonfly specimen displayed a range of 1.04‰, but only of 0.24‰ for $\delta^{66}\text{Zn}$. $\delta^{26}\text{Mg}$ 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 $\delta^{66}\text{Zn}$ 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 $\delta^{26}\text{Mg}$, but only of 0.17‰ for $\delta^{66}\text{Zn}$. $\delta^{26}\text{Mg}$ in stonefly 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 $\delta^{26}\text{Mg}$ and $\delta^{13}\text{C}$ values in *Kamimuria* spp. was observed (Appendix S2 Fig. S4).

DISCUSSION

Although differences in $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values among taxa with different feeding habits were evident, these differences were not reliably predicted by feeding habits. $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values in some taxa were in the range of those of metal sources. $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values in other taxa showed an offset to higher or lower $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values compared to those of metal sources indicating isotopic fractionation during Mg and Zn uptake. $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values were neither related to each other, nor to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values pointing to different elemental sources and/or other mechanisms driving isotopic variation. In the following, we firstly discuss the potential use of $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ 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, $\delta^{13}\text{C}$ values allow for estimating relative contributions of main dietary sources (periphyton vs. plant litter), while $\delta^{15}\text{N}$ values allow for assessing $\delta^{15}\text{N}$ trophic positions in stream food webs (Middelburg 2014). We found $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}$ values of consumers were closer to $\delta^{13}\text{C}$ values of periphyton, which agrees with the scarcity of plant litter at lower reaches. Furthermore, the overall higher $\delta^{15}\text{N}$ values of predatory insects and fish compared to those of primary consumers agree well with the ^{15}N trophic enrichment.

We found that $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values in consumers were neither related to each other (Fig. 5), nor to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Appendix S2 Figs. S2, S3), suggesting that $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ cannot be simply used to assess relative contributions of plant litter and periphyton as main Mg and Zn sources, and that $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ cannot be related to trophic level. Similarly, $\delta^{66}\text{Zn}$ could not be related to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ 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 $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ 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 $\delta^{15}\text{N}$ values in POM and periphyton (Karube et al. 2010, Ishikawa et al. 2014, 2016). Although the stream water and periphyton $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values were similar between May and November, more frequent, for example, monthly variations in $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values that we did not capture with our sampling design could exist. These variations in isotopic composition of sources could have impacted $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values in organisms of different larval stages that may last from several weeks to a few years (Merritt et al. 2008).

The overall absence of significant differences in $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values between upper vs. lower reaches (except for $\delta^{26}\text{Mg}$ 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 $\delta^{26}\text{Mg}$ 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 $\delta^{66}\text{Zn}$ 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 $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, we were able to distinguish some taxa of different feeding habits (except predatory caddisfly Rhyacophilidae and stonefly nymphs) based on $\delta^{26}\text{Mg}$ values in aquatic insects (Fig. 2). As such, filter feeders that rely on fine particulate organic matter (fPOM) as their main diet had lower $\delta^{26}\text{Mg}$ 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 ^{26}Mg enrichment (Fig. 2).

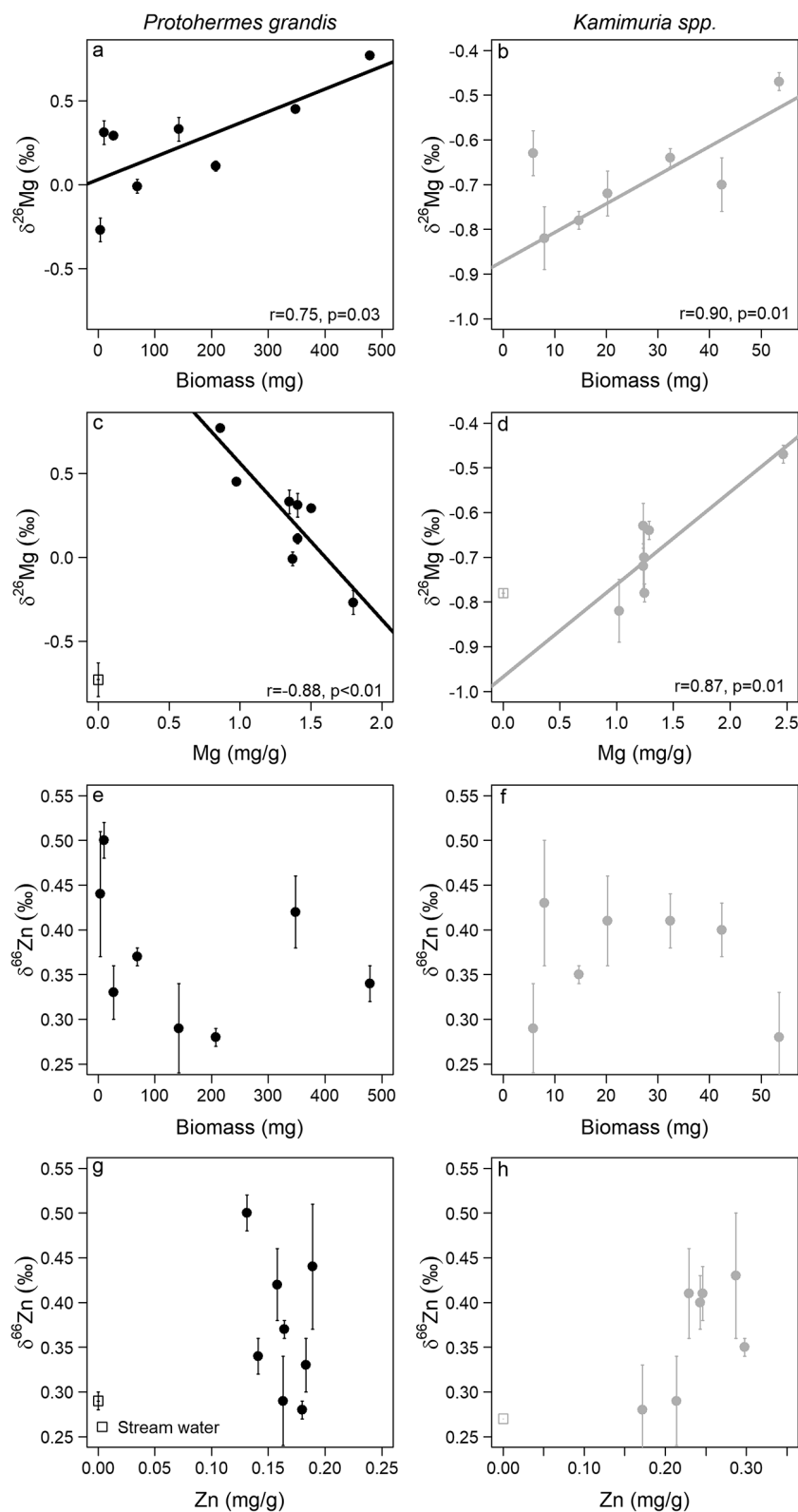


Fig. 6. Scatter plots of $\delta^{26}\text{Mg} \pm 2\sigma$ in dobsonflies (*Protohermes grandis*) of different sizes from Upper Ado in May 2018 against biomass (a) and against Mg concentration (c); of $\delta^{66}\text{Zn} \pm 2\sigma$ in dobsonflies against biomass (e)

(Fig. 6. *Continued*)

and against Zn concentration (g); of $\delta^{26}\text{Mg} \pm 2\sigma$ in stoneflies (*Kamimuria* spp.) of different sizes from Upper Yasu in May 2018 against biomass (b) and against Mg concentration (d); and of $\delta^{66}\text{Zn} \pm 2\sigma$ in stoneflies against biomass (f) and against Zn concentration (h). Open squares: stream water. Note that the *Kamimuria* spp. with the lowest biomass in (b) were not included in the correlation analysis between $\delta^{26}\text{Mg}$ vs. biomass.

Smaller predatory aquatic insects, that is, caddisflies and stoneflies, cannot be differentiated from filter feeders due to the similar range in $\delta^{26}\text{Mg}$ values. Collector–gatherers (Japanese freshwater crab) and omnivores (shrimp), both not aquatic insects, can be distinguished from other feeding guilds owing to their low $\delta^{26}\text{Mg}$ values. The ^{26}Mg enrichment in large predatory aquatic insects agrees with the ^{26}Mg 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 ^{26}Mg .

$\delta^{66}\text{Zn}$ patterns could not be generalized due to variations in $\delta^{66}\text{Zn}$ values of taxa across streams (Ado River vs. Yasu River), across locations (upper vs. lower), and given the small range in $\delta^{66}\text{Zn}$ values across taxa of different feeding habits within sampling locations. For instance, the similar $\delta^{66}\text{Zn}$ 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 $\delta^{66}\text{Zn}$ values although predatory fishes and dragonfly nymphs show an expected tendency to lower $\delta^{66}\text{Zn}$ values as observed for terrestrial and marine mammals (Jaouen et al. 2013, 2016a, b). Our data demonstrate the general importance of feeding habits on $\delta^{26}\text{Mg}$ values and partly on $\delta^{66}\text{Zn}$ 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 $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ 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 $\delta^{26}\text{Mg}$ 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 $\delta^{66}\text{Zn}$ values in Hydropsychidae spp. between stream water and periphyton suggest Zn in Hydropsychidae spp. is a mixture of these two sources. The even higher $\delta^{66}\text{Zn}$ 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. 2015) 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 $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values than plant litter pointing to the preferential incorporation of metabolically useful ^{26}Mg and ^{66}Zn , respectively, during dietary Mg and Zn uptake in the midgut. Our results agree with herbivory African mammals that had higher $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ 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 $\delta^{26}\text{Mg}$ 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 $\delta^{66}\text{Zn}$ 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 $\delta^{13}\text{C}$ 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 $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ 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 $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values in Rhyacophilidae larvae and in stream water pointed to metal uptake through anal papillae similar to filter-feeding caddisflies. $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values of stonefly nymphs (*Kamimuria* spp., *Oyamia* spp.) were often in the range of caddisfly larvae as potential prey, which agrees with our previous $\delta^{26}\text{Mg}$ data (Nitzsche et al. 2019) and similar $\delta^{66}\text{Zn}$ values between predatory stonefly nymphs and caddisfly larvae in a Canadian stream (Evans et al. 2016). To better understand potential mechanisms controlling $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ 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 $\delta^{26}\text{Mg}$ values of individuals with lower biomass were in the range of $\delta^{26}\text{Mg}$ 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 $\delta^{26}\text{Mg}$ with $\delta^{13}\text{C}$ 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 $\delta^{26}\text{Mg}$, biomass did not control $\delta^{66}\text{Zn}$ values highlighting the variability in $\delta^{66}\text{Zn}$ values of sources to individuals of different ages although smaller individuals tended to reflect the $\delta^{66}\text{Zn}$ values of stream water (Fig. 6 f,h). Consequently, $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ 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 $\delta^{26}\text{Mg}$ and $\delta^{66}\text{Zn}$ values given the lacking relationships with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Appendix S2 Fig. S4a,c). As even the smaller specimen showed an offset in ^{26}Mg relative to stream water (Fig. 6c), dobsonfly nymphs clearly fractionate Mg isotopes, possibly during intestinal Mg absorption from the diet in their midguts (Terra and Ferreira 2012). Furthermore, dobsonfly nymphs accumulated ^{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 ^{24}Mg assuming that ^{24}Mg is accumulated in the molted skin. In contrast to ^{26}Mg , the lacking correlations between $\delta^{66}\text{Zn}$ with biomass and with Zn concentration suggest the variability in $\delta^{66}\text{Zn}$ of sources, which could also comprise leaf-shredding crane fly larvae enriched in ^{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 ^{26}Mg enrichment, while $\delta^{66}\text{Zn}$ values suggest the variability in $\delta^{66}\text{Zn}$ of sources. Our data clearly show the difference between Mg and Zn sources and ^{26}Mg vs. ^{66}Zn fractionation among dobsonfly larvae.

Predatory dragonfly nymphs (Gomphidae spp.) tended to have higher $\delta^{26}\text{Mg}$ and lower $\delta^{66}\text{Zn}$ 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 $\delta^{26}\text{Mg}$ in bones and muscles, and $\delta^{66}\text{Zn}$ 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 $\delta^{26}\text{Mg}$ 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 Verboost 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}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.

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