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Stable nitrogen isotopic composition of amino acids reveals food web structure in stream ecosystems

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Running head: Amino acid $\delta^{15}N$ of stream animals

Abstract

The stable nitrogen isotopic composition of individual amino acids (SIAA) has recently been used to estimate trophic positions (TPs) of animals in several simple food chain systems. However, it is unknown whether the SIAA technique is applicable to more complex food web analysis. In this study we measured the SIAA of stream macroinvertebrates, fishes, and their potential food sources (periphyton and terrestrial C3 plant litter) collected from upper and lower sites in two streams having contrasting riparian landscapes. The stable nitrogen isotope ratios of glutamic acid and phenylalanine confirmed that for primary producers (periphyton and C3 litter) the TP was 1, and for primary consumers (e.g., mayfly and caddisfly larvae) was 2. We built a two-source mixing model to estimate the relative contributions of aquatic and terrestrial sources to secondary and higher consumers (e.g., stonefly larva and fishes) prior to the TP calculation. The estimated TPs (2.3-3.5) roughly corresponded to their omnivorous and carnivorous feeding habits, respectively. We found that the SIAA method offers substantial advantages over traditional bulk methods for food web analysis because the SIAA method defines the food web structure based on the metabolic pathway of amino groups, and the SIAA method can be used to estimate food web structure under conditions where the bulk method cannot be used for the analysis. Our result provides evidence that the SIAA method is applicable to the analysis of complex food webs, where heterogeneous resources are mixed.

Key Words: periphyton; terrestrial C3 litter; aquatic invertebrate; fish; two-source mixing model; resource reliance; trophic position; compound-specific isotope analysis; nitrogen metabolism

Introduction

The biological production fuels energy dynamics through an ecosystem (Lindeman 1942) via the trophic pathways composed of the prey-predator relationships involving spatial and temporal variations (Winemiller 1990). In most freshwater (e.g., stream) ecosystems associated with terrestrial and/or ocean ecosystems, biological production is supported by *in situ* primary production (e.g., periphytic algae attached to a substrate) as well as organic materials derived from other sources (e.g., terrestrial leaf litter) and these determine food web structure (Hynes 1970; Fisher and Likens 1973; Vannote et al. 1980; Nakano and Murakami 2001). Aquatic invertebrates are diverse animal consumers in stream food webs: such as algal grazing specialists (e.g., Heptageniidae larva: mayfly), leaf shredding specialists (e.g., Lepidostomatidae larva: caddisfly), and predatory generalists (e.g., Perlidae larva: stonefly) (Cummins 1973; Takemon 2005). The resource reliance of animals implies dynamic flow of material and energy among ecosystems (Baxter et al. 2005; Carpenter et al. 2005). Animals that have multiple dietary pathways (so-called omnivore) often dominate communities and occupy non-integer trophic positions, suggesting that in natural trophic networks the prey-predator relationships form a tangled food web rather than a simple food chain (Marczak et al. 2007; Thompson et al. 2007).

Analyses of the stable carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$, respectively) have contributed to the development of food web research during the last 30 years (e.g., Minagawa and Wada 1984; Fry 1991; Post et al. 2000). Animals' bulk-tissue $\delta^{13}C$ ($\delta^{13}C_{Bulk}$) and $\delta^{15}N$ ($\delta^{15}N_{Bulk}$) values have been used as indicators of food sources and trophic positions (TPs), respectively, because $\delta^{13}C$ values can distinguish primary producers (e.g., aquatic algae *vs*. terrestrial plants: Deines 1980), and $\delta^{15}N$ values increase with higher TP (e.g., Vander Zanden and Rasmussen 2001; Post 2002). Therefore, biplots for $\delta^{13}C_{Bulk}$ and

$\delta^{15}N_{Bulk}$ reveal food web structure in terms of resource importance and trophic pathways. However, in the stream ecosystems the $\delta^{13}C_{Bulk}$ of periphytic algae (primary producers) is sometimes too variable to enable assessment of the food sources for animals (Ishikawa et al. 2012), and for $\delta^{15}N_{Bulk}$ the isotope enrichment factor per trophic level (TL) of stream invertebrates is likely smaller and more variable than that of other animals (Bunn et al. 2013). To better understand the food web structure in stream ecosystems, a novel technique enabling analysis of food sources and TPs will be indispensable.

Techniques for measurement of the stable nitrogen isotopic composition of amino acids (SIAA) have recently been developed and applied to estimating the TPs of various animals (e.g., McClelland and Montoya 2002; Popp et al. 2007; Miller et al. 2013). In amino acid metabolism, glutamic acid is subject to deamination and transamination, which leads to increased isotope enrichment per TL (trophic enrichment factor: TEF = 8.0‰ in $\delta^{15}N$). In contrast, phenylalanine remains its amino group during metabolism because animals cannot synthesize phenylalanine themselves, resulting in little isotope enrichment per TL (TEF = 0.4‰ in $\delta^{15}N$) (Chikaraishi et al. 2009). The fairly constant TEFs in glutamic acid and phenylalanine have been observed in several systems, including feeding experiments performed by Chikaraishi et al. (2011) (quad-TLs: plant leaf > caterpillar and bee > wasp > hornet) and Steffan et al. (2013) (penta-TLs: apple leaves > apple aphid > hover fly > parasitoid > hyperparasitoid). Therefore, the TP of an animal in a single food chain can be determined using the following simple equation, with small deviations in TP estimates ($1\sigma \sim 0.2$) (Chikaraishi et al. 2009):

$$\mathrm{TP} = \frac{\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + \beta}{8.0 - 0.4} + 1 \qquad (1)$$

where $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ are the stable nitrogen isotope ratios of glutamic acid and phenylalanine of an animal, respectively. β is the difference between $\delta^{15}N_{Phe}$ and $\delta^{15}N_{Glu}$ for a primary producer (baseline) in the food chain (i.e., –3.4 for aquatic autotrophs; +8.4 for terrestrial C3 plants; Chikaraishi et al. 2009; 2010a; 2011). Thus, in a single food chain the TP of an animal can be estimated only from its $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values, without the data on the $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values of the baseline (Chikaraishi et al. 2009).

The applicability of the SIAA method to estimation of TPs has been tested for animals in simple ecosystems (e.g., a single food chain involving cabbage, caterpillar, and wasp: Chikaraishi et al. 2011). Few studies applying the SIAA method to complex food webs (e.g., where both aquatic- and terrestrial-derived resources potentially contribute to the diet of animals) have been reported (c.f., reconstruction of marine and terrestrial paleoenvironments: Naito et al. 2010). In stream food webs where aquatic and terrestrial resources are mixed, the proportion of resources derived from aquatic and terrestrial food chains can be used in the estimation of the TP of animals (e.g., macroinvertebrates and fishes), because aquatic and terrestrial primary producers have distinctive β values in Eq. 1. In this study we test the applicability of the SIAA method for analyzing stream food webs, with assumption of constant TEFs in $\delta^{15}N_{Glu}$ (8.0‰) and $\delta^{15}N_{Phe}$ (0.4‰) (Chikaraishi et al. 2009) for stream invertebrates and fishes. We build a two-source mixing model using the $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values of periphyton, C3 litter, and animals to estimate both resource importance and trophic pathways in stream food webs.

Materials and methods

Study sites and sample collection

In November (winter) 2011 and May (summer) 2012, stream macroinvertebrates, fishes, and

their potential food sources (periphyton and terrestrial C3 litter) were collected from upper and lower sites of the Yasu River and the Ado River, central Japan (Table A1, Fig. A1, A2). The Yasu River is the largest watershed in the Lake Biwa basin: the upper site is pristine while the lower site is affected by urban development. The concentration and isotope value of nitrate increase in the downstream direction in the Yasu River (Ohte et al. 2010). The Ado River is the third largest watershed in the Lake Biwa basin. The natural landscape has been retained throughout its length, and the concentration and isotope value of nitrate do not greatly change along its course in the Ado River (Ohte et al. 2010). Several plants with C3 photosynthesis (Cupressaceae and Fagaceae) dominate the riparian vegetation at each of the study sites.

Aquatic invertebrates and fishes were collected at each site using a hand net. We also randomly collected several submerged river cobbles, which were rinsed gently with distilled water prior to collecting the periphyton from the cobble surface, using a brush and distilled water. The resulting slurry was placed into a 100-mL polypropylene bottle (3-5 replicates per site). The terrestrial C3 litter (hereafter, C3 litter) comprising C3 plants (mainly Fagaceae and Ericaceae), was collected from several leaf packs within the stream at each site: the exception was the lower site of the Yasu River in November, where no leaf packs were present: on this occasion, rather than C3 litter we collected particulate organic material (POM) using a surber net (mesh size 1000 μm) placed vertically in the current in the center of the channel. Neither C3 litter nor POM included C4 plants. All samples were held on ice in the dark until further processing in the laboratory. Gut contents of the invertebrates were not eliminated because some of them had been already dead during transportation. We identified and categorized invertebrates into functional feeding groups (FFGs: grazer; shredder; filter feeder; predator; and other invertebrates). Isotope measurements were based on single invertebrates where the body size was large enough for analysis (i.e., > 3.0 mg dry weight per

individual), or were based on several individuals belonging to the same family, which were combined to form the sample for analysis. All samples were freeze-dried, and each was ground into a fine powder prior to analysis.

Bulk stable carbon and nitrogen isotope measurements

We measured the bulk stable carbon and nitrogen isotope ratios ($\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$, respectively) of periphyton, C3 litter, invertebrates, and fishes. Each sample was packed into a tin capsule, and the $\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$ (‰) were measured using a Flash EA1112 elemental analyzer connected to a Delta XP isotope ratio mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a Conflo III interface (Thermo Fisher Scientific). The $\delta^{13}C$ and $\delta^{15}N$ values were reported relative to that of Vienna Pee Dee Belemnite (VPDB) and atmospheric N_2 (Air), respectively. Data were corrected using internal standards (CERKU-01 DL-Alanine: $\delta^{13}C_{VPDB}$ = –25.36‰, $\delta^{15}N_{Air}$ = –2.89‰; CERKU-02 L-Alanine: $\delta^{13}C_{VPDB}$ = –19.04‰, $\delta^{15}N_{Air}$ = +22.71‰; CERKU-03 Glycine: $\delta^{13}C_{VPDB}$ = –34.92‰, $\delta^{15}N_{Air}$ = +2.18‰) that were corrected to multiple international standards (Tayasu et al. 2011). The standard deviations of the $\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$ measurements were within 0.10‰ and 0.14‰, respectively.

Amino acid purification and stable nitrogen isotope measurement

For compound-specific isotope analysis, amino acids in all samples were purified by HCl hydrolysis followed by *N*-pivaloyl/isopropyl (Pv/iPr) addition, according to the improved procedures of Chikaraishi et al. (2007). In brief, samples of animals (~3 mg) and periphyton, POM, and C3 litter (~20 mg) were hydrolyzed in 12 mol L^{-1} HCl at 110 °C for 12 h. The hydrolysates were filtrated through a pipette stuffed with quartz wool, washed with *n*-hexane/dichloromethane (3:2, v/v) to remove large particles and hydrophobic constituents

(e.g., lipids), respectively, and evaporated to dryness under a N_2 stream. After derivatization with thionyl chloride/2-propanol (1:4, v/v) at 110 °C for 2 h and pivaloyl chloride/dichloromethane (1:4, v/v) at 110 °C for 2 h, and liquid-liquid extraction with 0.5 ml of *n*-hexane/dichloromethane (3:2, v/v) and 0.2 ml of distilled water, the Pv/iPr derivatives of amino acids were dissolved in dichloromethane.

We measured the stable nitrogen isotopic composition of amino acids following the modified method of Chikaraishi et al. (2010b). Briefly, the $\delta^{15}N$ values of the individual amino acids were determined by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) using a Delta V plus isotope ratio mass spectrometer (Thermo Fisher Scientific) coupled to a gas chromatograph (GC7890A; Agilent Technologies, Santa Clara, CA, USA) via a modified GC-Isolink interface consisting of combustion and reduction furnaces. The amino acid derivatives were injected into the GC column using a Gerstel PTV injector in solvent vent mode. The PTV temperature program was as follows: 50 °C (initial temperature) for 0.25 min, heating from 50 °C to 270 °C at the rate of 600 °C min^{-1}, isothermal hold at 270 °C for 10 min. The combustion was performed in a microvolume ceramic tube with CuO, NiO, and Pt wires at 1030 °C, and the reduction was performed in a microvolume ceramic tube with reduced Cu wire at 650 °C. The GC was equipped with an Ultra-2 capillary column (50 m, 0.32 mm i.d., 0.52 μm film thickness; Agilent Technologies). The GC oven temperature was programmed as follows: initial temperature 40 °C for 2.5 min, increase at 15 °C min^{-1} to 110 °C, increase at 3 °C min^{-1} to 150 °C, increase at 6 °C min^{-1} to 220 °C, hold at the final temperature for 14 min. The carrier gas (He) flow rate through the GC column was 1.4 ml min^{-1}. The CO_2 generated in the combustion furnace was removed using a liquid nitrogen trap. Standard mixtures of at least 5 amino acids ($\delta^{15}N$ ranging from –6.27 to +22.71‰) were analyzed every 1-6 samples to confirm the reproducibility of the isotope measurements. Analytical errors (1 σ) of the standards were better than 0.7‰ with a

minimum sample quantity of 60 ng N.

Estimation of periphyton contribution and trophic position

Two-isotope and two-source mixing models are widely used in various ecological studies including food web research (e.g., Fry 2006). Using $\delta^{15}N_{Bulk}$ and $\delta^{13}C_{Bulk}$ values of periphyton (average of 3-5 replicates), C3 litter, and animals at each site, the local periphyton contributions to animals relative to C3 litter (f) were calculated using Eq. 2 (see Appendix for more details on algebraic procedures):

$$f = \frac{\frac{\delta^{15}N_{Bulk}[A] - \delta^{15}N_{Bulk}[L]}{\Delta_N} - \frac{\delta^{13}C_{Bulk}[A] - \delta^{13}C_{Bulk}[L]}{\Delta_C}}{\frac{\delta^{15}N_{Bulk}[P] - \delta^{15}N_{Bulk}[L]}{\Delta_N} - \frac{\delta^{13}C_{Bulk}[P] - \delta^{13}C_{Bulk}[L]}{\Delta_C}} \quad (2)$$

where $0 \leq f \leq 1$ and $\delta^{15}N_{Bulk}[A]$, $\delta^{13}C_{Bulk}[A]$, $\delta^{15}N_{Bulk}[L]$, $\delta^{13}C_{Bulk}[L]$, $\delta^{15}N_{Bulk}[P]$, and $\delta^{13}C_{Bulk}[P]$ are $\delta^{15}N_{Bulk}$ and $\delta^{13}C_{Bulk}$ of animal [A], those of C3 litter [L], and those of periphyton [P] in each site, respectively. Δ_N and Δ_C are trophic enrichment factors for $\delta^{15}N_{Bulk}$ (3.4‰) and $\delta^{13}C_{Bulk}$ (0.8‰), respectively (Vander Zanden and Rasmussen 2001). Using Eq. 2, the TPs of animals were estimated according to Eq. 3:

$$TP = \frac{\delta^{15}N_{Bulk}[A] - \delta^{13}C_{Bulk}[A] - \{f(\delta^{15}N_{Bulk}[P] - \delta^{13}C_{Bulk}[P]) + (1-f)(\delta^{15}N_{Bulk}[L] - \delta^{13}C_{Bulk}[L])\}}{\Delta_N - \Delta_C} + 1 \quad (3)$$

Using the $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values of periphyton (average of 3-5 replicates), C3 litter, and animals at each site, the local periphyton contributions to animals relative to C3

litter (g) were calculated in the same manner:

$$g = \frac{\frac{\delta^{15}N_{Glu}[A] - \delta^{15}N_{Glu}[L]}{\Delta_{Glu}} - \frac{\delta^{15}N_{Phe}[A] - \delta^{15}N_{Phe}[L]}{\Delta_{Phe}}}{\frac{\delta^{15}N_{Glu}[P] - \delta^{15}N_{Glu}[L]}{\Delta_{Glu}} - \frac{\delta^{15}N_{Phe}[P] - \delta^{15}N_{Phe}[L]}{\Delta_{Phe}}} \quad (4)$$

where $0 \leq g \leq 1$ and $\delta^{15}N_{Glu}[A]$, $\delta^{15}N_{Phe}[A]$, $\delta^{15}N_{Glu}[L]$, $\delta^{15}N_{Phe}[L]$, $\delta^{15}N_{Glu}[P]$, and $\delta^{15}N_{Phe}[P]$ are $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ of animal [A], those of C3 litter [L], and those of periphyton [P] in each site, respectively. Δ_{Glu} and Δ_{Phe} are trophic enrichment factors for $\delta^{15}N_{Glu}$ (8.0‰) and $\delta^{15}N_{Phe}$ (0.4‰), respectively (Chikaraishi al. 2009). Using Eq. 4, the TPs of animals were estimated according to Eq. 5:

$$TP = \frac{\delta^{15}N_{Glu}[A] - \delta^{15}N_{Phe}[A] - \{g(\delta^{15}N_{Glu}[P] - \delta^{15}N_{Phe}[P]) + (1-g)(\delta^{15}N_{Glu}[L] - \delta^{15}N_{Phe}[L])\}}{\Delta_{Glu} - \Delta_{Phe}} + 1 \quad (5)$$

Animals for which the periphyton contributions were calculated to be > 100% or < 0% were removed from the analysis (7 of a total of 87 data points). Data on C3 litter were not available for the lower site of the Yasu River in November and consequently the TPs of animals at this site were not calculated (11 of a total of 87 data points). All statistical analyses and graphing were performed using R 2.14.2 software (R Development Core Team 2012), with the significance level set $\alpha = 0.01$.

Results

Bulk stable carbon and nitrogen isotope ratios

Analysis of variance showed that the $\delta^{15}N_{Bulk}$ values of periphyton were significantly different between the two sites (upper sites *vs*. lower sites; $p < 0.001$), but were not different between the two seasons (November *vs*. May; $p = 0.14$) or between the two rivers (Yasu *vs*. Ado; $p = 0.20$). In both November and May the $\delta^{15}N_{Bulk}$ values of periphyton in the Yasu River were significantly lower at the upper site (–2.4 ± 0.76‰, mean ± 1 standard deviation, $n = 7$) than the lower site (+5.9 ± 1.95‰, $n = 8$) (Tukey's HSD, $p < 0.001$ in both seasons). In contrast, the $\delta^{15}N_{Bulk}$ values of periphyton in the Ado River were not significantly different between the upper site (+0.5 ± 0.68‰, $n = 9$) and the lower site (+1.7 ± 0.45‰, $n = 8$) (Tukey's HSD, $p = 0.35$ in November and $p = 0.10$ in May; Fig. 1, 2). The $\delta^{13}C_{Bulk}$ values of periphyton showed large intra-site variations (5-10‰) in all sites, while those of the C3 litter remained relatively constant among sites (ca. –30‰) (Fig. 1, 2). For animals, the $\delta^{13}C_{Bulk}$ values fell mostly between the $\delta^{13}C_{Bulk}$ values of periphyton and C3 litter. An exception was the lower site of the Ado River in November, where the $\delta^{13}C_{Bulk}$ values of some animals were higher than those of periphyton (Fig. 1). The $\delta^{15}N_{Bulk}$ values of invertebrates fell mostly between the $\delta^{15}N_{Bulk}$ values of primary producers (i.e., periphyton and C3 litter) and fishes. An exception was the lower site of the Yasu River in November, where the $\delta^{15}N_{Bulk}$ values of periphyton were higher than those of invertebrates (Fig. 1). Overall, the amount of animals' $\delta^{15}N_{Bulk}$ and $\delta^{13}C_{Bulk}$ data that could be used for calculation of two-source mixing model was larger in May (31 of a total of 37 data points) than in November (20 of a total of 36 data points).

Primary producers

Analysis of variance showed that the $\delta^{15}N_{Phe}$ values of periphyton were significantly different between the two sites ($p < 0.001$), but were not different between the two seasons ($p = 0.10$) or between the two rivers ($p = 0.04$). In both November and May, the $\delta^{15}N_{Phe}$ values of

periphyton in the Yasu River were significantly lower at the upper site (–4.2 ± 1.80‰, n = 6) than the lower site (+4.8 ± 2.52‰, n = 8) (Tukey's HSD, $p < 0.001$ in both seasons). In contrast, the $\delta^{15}N_{Phe}$ values of periphyton in the Ado River were not significantly different between the upper site (–1.4 ± 1.92‰, n = 8) and the lower site (–0.9 ± 1.05‰, n = 8) (Tukey's HSD, $p > 0.99$ in both seasons; Fig. 3, 4). The differences between the $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values of periphyton were relatively constant (+3.7 ± 1.69‰, n = 30), and not significantly different from those reported for aquatic primary producers (Chikaraishi et al. 2009: +3.4 ± 0.9‰, n = 25) (Wilcoxon test: W = 327, p = 0.42). However, the differences between the $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values of the C3 litter (–10.7 ± 1.31‰, n = 7) were significantly different from those reported for terrestrial C3 plants (Chikaraishi et al. 2010a: –8.4 ± 1.6‰, n = 17) (Wilcoxon test: W = 104, p = 0.005). The difference between $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values of POM collected from the lower site of the Yasu River on November (+7.4‰) was higher than those of aquatic primary producers (+3.4‰) and terrestrial C3 plants (–8.4‰) (Fig. 3c), indicating that POM included not only primary producers, but also living and/or dead heterotrophs.

Primary consumers

The $\delta^{15}N_{Phe}$ values of primary consumers (mayfly and caddisfly larvae; an exception was the larvae of the leaf shredding caddisfly *Lepidostoma japonicum*) in the Yasu River were much lower at the upper site (–4.5 ± 2.57‰, n = 5) than the lower site (+6.2 ± 2.35‰, n = 7), while in the Ado River the $\delta^{15}N_{Phe}$ values of primary consumers were slightly lower at the upper site (–0.2 ± 1.64‰, n = 7) than the lower site (+1.1 ± 0.59‰, n = 7). For grazing mayflies (larvae of Heptageniidae spp. and *Baetis* spp.) the $\delta^{15}N_{Glu}$ values were approximately 8‰ higher than those of local periphyton while the $\delta^{15}N_{Phe}$ values were similar to the periphyton values, and thus they were located near the line of aquatic TL = 2 (Fig. 3, 4). The two-source mixing

model showed that the reliance of mayflies on periphyton was 90 ± 6.5% (*n* = 9; Fig. 5a) with the TP of 2.1 ± 0.08 (*n* = 9; Fig. 5b). The $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values of filter feeding caddisflies (larvae of Hydropsychidae spp. and *Stenopsyche marmorata*) showed large variations among sites and seasons, but their reliance on periphyton (87 ± 3.3%, *n* = 8) and TP (2.2 ± 0.14, *n* = 8) were less variable than other animals (Fig. 5). The $\delta^{15}N_{Phe}$ values of larvae of the leaf shredding caddisfly *L. japonicum* were 10-15‰ higher than those of local periphyton, and were similar to that of C3 litter. The periphyton contribution to shredders was thus estimated to be 24 ± 16.9% (*n* = 5, Fig. 5a) with the TP of 2.0 ± 0.27 (*n* = 5, Fig. 5b).

Secondary consumers and fishes

The $\delta^{15}N_{Glu}$ values of secondary consumers were similar to those of grazers and filter feeders (Fig. 5). As with the primary consumers, the $\delta^{15}N_{Phe}$ values of secondary consumers (i.e., predatory larvae: the dragonfly Gomphidae spp.; the stoneflies *Kamimuria tibialis*, Chloroperlidae spp., *Paragnetina tinctipennis*, *Oyamia lugubris*, *Niponiella limbatella*; and the dobsonfly *Protohermes grandis*) in the Yasu River were much lower at the upper site (–0.9 ± 1.09‰, *n* = 5) than the lower site (+6.3 ± 1.61‰, *n* = 7), while in the Ado River there was only a small difference between the upper site (+1.3 ± 0.94‰, *n* = 15) and the lower site (+1.5 ± 1.39‰, *n* = 7). Dragonfly, stoneflies, and dobsonfly were 85 ± 8.5% (*n* = 4), 81 ± 9.0% (*n* = 18), and 82 ± 10.0% (*n* = 5) reliant on periphyton, respectively (Fig. 5a). The TPs of predators (dragonfly: 2.3 ± 0.10; stoneflies: 2.5 ± 0.25; dobsonfly: 2.3 ± 0.18) were higher than those of primary consumers, but were < 3 (Fig. 5b). Larvae of the crane fly (Tipulidae spp., FFG not specified) were 70 ± 9.0% (*n* = 4; Fig. 5a) reliant on periphyton with the TP of 2.5 ± 0.23 (*n* = 4; Fig. 5b). Fishes, including demersal goby (*Rhinogobius* spp.) and other fishes (trout, chub, and minnow) were 77 ± 8.0% (*n* = 10) and 78 ± 10.9% (*n* = 6) reliant on periphyton, respectively (Fig. 5a). The TPs in our dataset were highest for fishes (Fig. 5b), including for

goby (3.1 ± 0.28, n = 10) and the other fishes (2.8 ± 0.25, n = 6).

The amount of animals' $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ data that could be used for calculation of two-source mixing model was similar between November (36 of a total of 39 data points) and May (33 of a total of 37 data points). Analysis of variance showed that the periphyton contributions (relative to the C3 litter) to animals were significantly different between the two seasons and the two rivers, and among animal groups, but were not significantly different between the two sites (Table A2). Periphyton contribution percentage in the Yasu River and May were significantly lower than in the Ado River and November (Tukey's HSD, $p < 0.001$). The TPs of animals were significantly different between seasons, sites (marginally), and among animal groups, but were not significantly different between rivers (Table A3). The TPs of animals in November were significantly lower than those in May (Tukey's HSD, $p < 0.01$).

Comparisons between bulk and SIAA methods

Based on Eq. 2-5, TPs estimated from $\delta^{15}N_{Bulk}$ and $\delta^{13}C_{Bulk}$ values and from $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values were compared and a different pattern was observed between November and May (Fig. 6). The amount of data for November was small because the $\delta^{15}N_{Bulk}$ and $\delta^{13}C_{Bulk}$ values of periphyton were too variable to construct a two-source mixing model for estimating the relative contributions of periphyton and C3 litter to animals (Fig. 1): approximately 50% of the data points for animals were removed from the analysis because the estimated periphyton contributions exceeded 100%. Furthermore, the bulk estimated TPs for November were different from the SIAA estimated TPs: the SIAA estimated TPs ranged from 2 to 3, while the bulk estimated TPs varied widely from 1 to 4 (Fig. 6a). On the other hand, as the $\delta^{13}C_{Bulk}$ values of animals for May were between those of periphyton and C3 litter, and the $\delta^{15}N_{Bulk}$ values of animals were higher than those of periphyton and C3 litter (Fig. 2), in most cases the periphyton contribution to animals, and their TPs, were estimated. The TPs for May,

estimated using the bulk and SIAA methods, were more alike than those for November, although for several primary consumers (grazers and shredders) the bulk method provided TP estimates < 2 (Fig. 6b).

Discussion

The stable nitrogen isotopic composition of amino acids (SIAA) is useful for understanding the structure of stream food webs: this conclusion was induced by comparing the resource reliance and trophic positions determined using bulk and SIAA methods for a range of variable stream conditions (upper *vs.* lower parts of the streams; pristine *vs.* urbanized landscapes; and summer *vs.* winter). One important assumption of the linear mixing model based on $\delta^{15}N_{Bulk}$ and $\delta^{13}C_{Bulk}$ values is that dietary nitrogen and carbon are assimilated by animals in the same proportions (Phillips and Koch 2002), although the C:N ratios of animals and those of their diets are not necessarily identical in natural food webs (Post 2002). The SIAA method does not rely on this assumption because the biplot for $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ defines the food web structure based on the metabolic pathway of amino groups.

Our seasonal data showed two contrasting results for the bulk methods. The $\delta^{15}N_{Bulk}$ and $\delta^{13}C_{Bulk}$ values for May were able to estimate relative contributions of periphyton and C3 litter to animals, and the bulk estimated TPs were well correlated with the SIAA estimated TPs (Fig. 6b), suggesting that both methods are applicable to stream food web analysis. However, the bulk method was not applicable to analyzing stream food webs in November, because the $\delta^{15}N_{Bulk}$ values of some animals were lower than those of periphyton (e.g., Lower Yasu; Fig. 1), and because the $\delta^{13}C_{Bulk}$ values of some animals were not between those of periphyton and C3 litter (e.g., Lower Ado; Fig. 1). As noted in many reports, variations in enrichment of $\delta^{15}N_{Bulk}$ among taxa and variations in the $\delta^{13}C_{Bulk}$ values of periphyton may

have caused problems in the analysis of stream food webs (McCutchan et al. 2003; Dekar et al. 2009; Ishikawa et al. 2012; Bunn et al. 2013). In November, the bulk estimated TPs were not consistent with the SIAA estimated TPs, and the former provided contradictory results in some animals (e.g., the TPs of some invertebrates were < 2, Fig. 6a). In contrast, our results using the SIAA method met the assumptions that the $\delta^{15}N_{Glu}$ values of animals are higher than those of primary producers, and that the $\delta^{15}N_{Phe}$ values of animals fall between those of periphyton and C3 litter (Fig. 3, 4). The results indicate that both periphyton and C3 litter support stream food webs, and that animals at higher trophic positions integrate aquatic and terrestrial food chains.

The $\delta^{15}N_{Phe}$ values of periphyton were variable among sites, probably reflecting *in situ* nutrient conditions (Pastor et al. 2013). In the Yasu River the $\delta^{15}N_{Bulk}$ and $\delta^{15}N_{Phe}$ values of periphyton were higher at the lower site than the upper site, but this was not the case for the Ado River. The result is consistent with the pattern of elevation of $\delta^{15}N\text{-}NO_3$ along the Yasu River reflecting anthropogenic nitrogen loading in the urbanized watershed (Ohte et al. 2010). As the $\delta^{15}N_{Phe}$ values of primary producers reflect the $\delta^{15}N$ of inorganic nitrogen (e.g., $\delta^{15}N\text{-}NO_3$) (Chikaraishi et al. 2009), the intra-site variation in $\delta^{15}N_{Phe}$ values of periphyton suggests that either $\delta^{15}N$ of inorganic nitrogen or fractionation between inorganic nitrogen and algae vary within a site. On the other hand, the $\delta^{15}N_{Phe}$ values of C3 litter were much higher than those of periphyton, and corresponded to or were below the terrestrial C3 baseline (TL = 1), expected on the basis of the results of Chikaraishi et al. (2010a; 2011). Terrestrial C3 plants synthesize lignin from phenylalanine through the phenylpropanoid pathway, but aquatic autotrophs do not (Bender 2012). Kinetic isotope fractionation from phenylalanine to lignin may result in elevated $\delta^{15}N_{Phe}$ values relative to $\delta^{15}N$ values of other amino acids (e.g., glutamic acid) in terrestrial C3 plants, and consequently relative to $\delta^{15}N_{Phe}$ values of aquatic autotrophs. Our results suggest that both aquatic and terrestrial primary producers have large

395 $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ variations as several previous studies have shown (e.g., Chikaraishi et al.
396 2009, 2011; Naito et al. 2013). Further studies will be necessary to elucidate what controls the
397 large variations in the $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values of primary producers in different
398 environments.

399 The $\delta^{15}N_{Glu}$ values of grazers were approximately 8.0‰ higher than those of
400 periphyton while the $\delta^{15}N_{Phe}$ values of both were similar, suggesting that grazing animals
401 occupy the position of TL = 2 in the aquatic food chain. On the other hand, the $\delta^{15}N_{Phe}$ values
402 of shredders were slightly lower than those of C3 litter, suggesting that leaf shredding animals
403 are partly subsidized by $^{15}N_{Phe}$-depleted aquatic resources. The two-source mixing model
404 indicated that the periphyton contribution to predators was less than that to grazers,
405 suggesting that predators rely on both aquatic and terrestrial resources. It also indicated that
406 the TPs of predators were higher than those of grazers and shredders, but were < 3, suggesting
407 that the larvae of dragonfly, stonefly, and dobsonfly are not completely carnivores, but are
408 partly omnivores. This result is consistent with previous gut content analysis showing that the
409 larvae of two stoneflies (*O. lugubris* and *K. tibialis*) feed on both animals and algae
410 (Miyasaka and Genkai-Kato 2009). In contrast, as the larvae of dragonfly and dobsonfly have
411 highly specialized mouthparts for eating animal prey, and their guts include animals
412 exclusively (Hayashi 1988; Takemon 2005), our TP estimates of dragonfly and dobsonfly
413 larvae were lower than those predicted based on diet. In most cases the TPs of fishes were > 2
414 but < 3, suggesting that their diet includes autotrophs and heterotrophs derived from both
415 aquatic and terrestrial food webs, and that they assimilate both animal- and plant-derived
416 proteins.

417 In this study we assumed constant TEFs in $\delta^{15}N_{Glu}$ (Δ_{Glu} = 8.0‰) and $\delta^{15}N_{Phe}$ (Δ_{Phe}
418 = 0.4‰) for stream invertebrates and fishes, based on the metabolic theory of amino acids and
419 several empirical observations (Chikaraishi et al. 2009; 2011; Steffan et al. 2013). The results

suggested that this assumption is reasonable for primary consumers (i.e., grazers and shredders), while it should be examined for secondary and higher consumers (e.g., the larvae of dragonfly and dobsonfly) in further studies. Indeed, the value of $\Delta_{Glu} - \Delta_{Phe}$ is reported as lower than 7.6‰ between some animals and their potential food sources (e.g., penguin: 3.4-3.8‰, Lorrain et al. 2009; stingray and shark: 5.0 ± 0.6‰, Dale et al. 2011). In addition, a feeding experiment indicated that the value of $\delta^{15}N_{Glu} - \delta^{15}N_{Phe}$ in harbor seal is only 4.3‰ higher than the value of their exclusive diet (wild herring) (Germain et al. 2013).

The seasonal differences in periphyton contributions to animals suggest that high in-stream production in summer and/or large inputs of terrestrial resources in winter are reflected in the biomass of animals (Nakano and Murakami 2001). The TPs of animals were also slightly different between seasons, probably because the predator species analyzed were different between November and May: for example, the dominant stoneflies were *K. tibialis* in November (TP = 2.3 ± 0.19; N = 8), but were *N. limbatella* in May (TP = 2.6 ± 0.30; N = 6). We did not expect that the periphyton contributions would be lower in the Yasu River than in the Ado River, because the watershed of the former is more urbanized and has a higher dissolved nitrate concentration (Ohte et al. 2010), which would increase in-stream primary production. In addition, we did not find a significant difference in the periphyton contributions between upper and lower sites, suggesting that nitrogen transfer pathway in food webs does not greatly change along a river continuum.

Most ecosystems are open, and the movement of materials and energy among ecosystems plays an important role in several ecological processes (e.g., the addition of extra resources make food webs more complex: Polis et al. 1997; Nakano and Murakami 2001). Although the number of studies using the SIAA method for estimating the TPs of animals has recently increased (e.g., McClelland and Montoya 2002; Popp et al. 2007; Miller et al. 2013), these studies have been limited to simple food chain systems (to our knowledge exceptions

445 are a few archaeological studies; Naito et al. 2010; 2013; Styring et al. 2010) because aquatic
446 and terrestrial primary producers have distinctive $\delta^{15}N$ differences between source amino
447 acids (e.g., phenylalanine) and trophic amino acids (e.g., glutamic acid) (Chikaraishi et al.
448 2009; 2010a). We overcome this limitation by applying a two-source mixing model to stream
449 food webs involving mixed aquatic and terrestrial resources. Our data suggest novel
450 applications of the SIAA method in addition to estimating the TPs of animals, assessing the
451 relative contributions of aquatic and terrestrial resources to animals (Fig. 7): this structure is
452 central to understanding how aquatic and terrestrial food chains are incorporated into stream
453 ecosystems. Furthermore, amino acids are fundamental to the transfer of nitrogen within and
454 among ecosystems (Bender 2012). Based on these advantages, we conclude that a mixing
455 model using the SIAA method can provide useful information for the analysis of complex
456 food webs and nitrogen cycling in natural ecosystems.
457

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Stable nitrogen isotopic composition of amino acids reveals food web structure in stream ecosystems

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Running head: Amino acid $\delta^{15}N$ of stream animals

Figure legends

Fig. 1.

Biplot for the bulk stable carbon and nitrogen isotope ratios ($\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$, respectively) of animals and their potential food sources collected in November 2011. Filled diamonds and squares are periphyton and terrestrial C3 litter, respectively. A cross surrounded by a square in Lower Yasu indicates particulate organic material (POM). Open diamond: grazer; open square: shredder; open circle: filter feeder; open triangle: predator; and open reverse-triangle: other invertebrates. Filled and open stars are demersal fish (goby) and other fishes, respectively

Fig. 2.

Biplot for the bulk stable carbon and nitrogen isotope ratios ($\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$, respectively) of animals and their potential food sources collected in May 2012. The symbols are the same as described in Fig. 1

Fig. 3.

Biplot for the stable nitrogen isotope ratios of glutamic acid ($\delta^{15}N_{Glu}$) and phenylalanine ($\delta^{15}N_{Phe}$) of animals and their potential food sources, collected in November 2011. Aquatic and terrestrial baselines (TL = 1) are indicated as solid lines (aquatic: $\delta^{15}N_{Glu} - \delta^{15}N_{Phe} = +3.4$; terrestrial C3: $\delta^{15}N_{Glu} - \delta^{15}N_{Phe} = -8.4$; Chikaraishi et al. 2009, 2010). Stepwise enrichments of $\delta^{15}N_{Glu}$ (+8.0‰) and $\delta^{15}N_{Phe}$ (+0.4‰) along with trophic levels are shown as dashed (TL = 2) and dotted (TL = 3) lines for both aquatic and terrestrial food chains. The symbols are the same as described in Fig. 1

Fig. 4.

Biplot for the stable nitrogen isotope ratios of glutamic acid ($\delta^{15}N_{Glu}$) and phenylalanine ($\delta^{15}N_{Phe}$) of animals and their potential food sources, collected in May 2012. The symbols are the same as described in Fig. 1 and 3

Fig. 5.

a) Periphyton contribution to animals relative to terrestrial C3 litter (%), estimated using a SIAA based two-source mixing model (see Eq. 4). Periphyton contribution to periphyton (*n* = 13) and C3 litter (*n* = 7) were fixed at 100% and 0%, respectively. Grazer: G (*n* = 9); predator: P (dragonfly: *n* = 4; stonefly: *n* = 18; dobsonfly: *n* = 5); other invertebrates: O (*n* = 4); filter feeder: F (*n* = 8); shredder: S (*n* = 5); goby (*n* = 10); and other fishes (*n* = 6); and b) Trophic position of animals based on the mixing proportion of aquatic (periphyton) and terrestrial (C3 litter) resources estimated using a SIAA based two-source mixing model (see Eq. 5). The box and bar depict inter-quartile (Q1 and Q3) and median, respectively. The whisker represents the most extreme data point that is no more than 1.5-fold the inter-quartile range. Outliers are shown where applicable

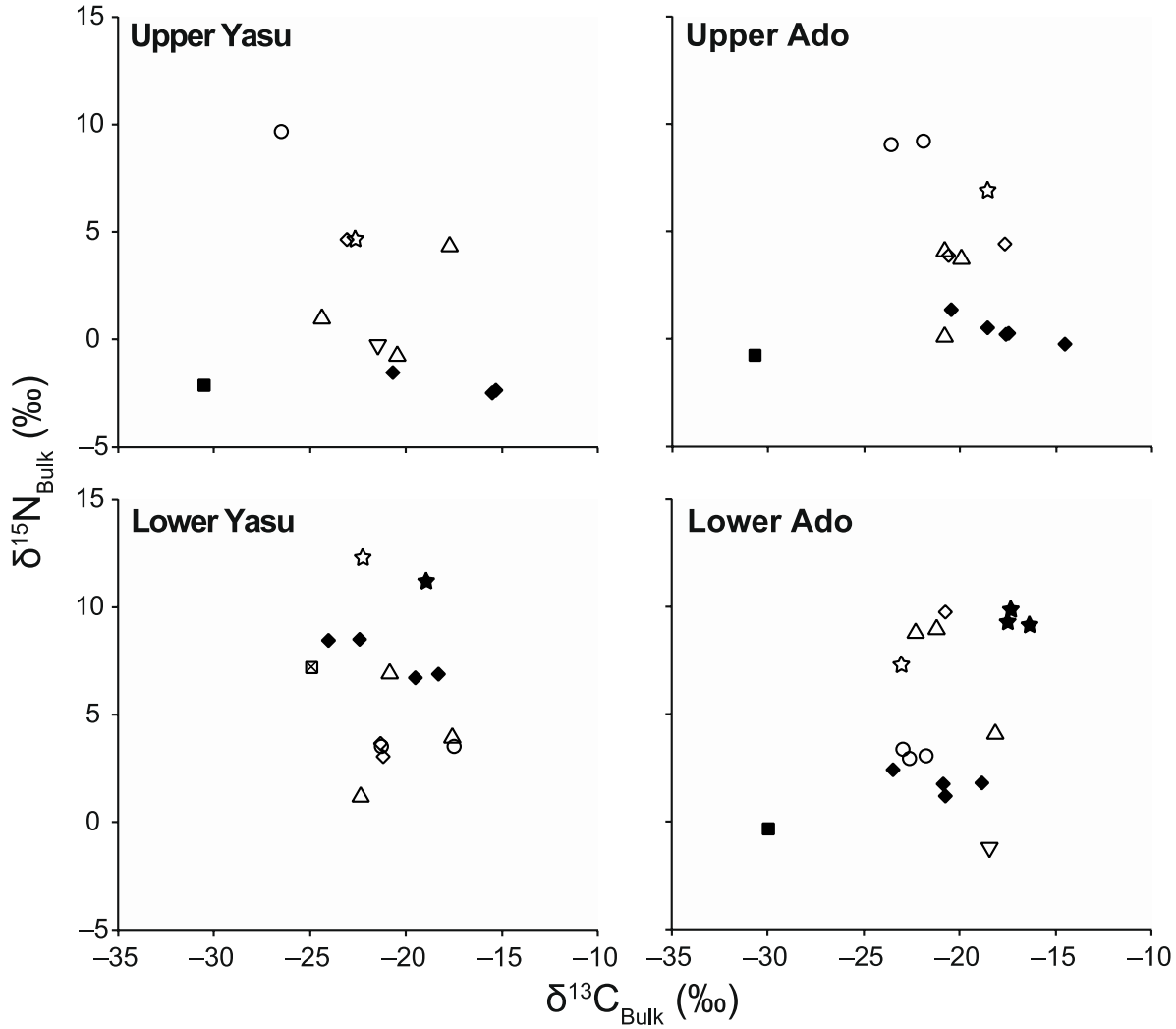
Fig. 6.

Biplot for the trophic positions estimated using the bulk method (Eq. 2-3) *vs*. those estimated using the SIAA method (Eq. 4-5) in a) November 2011 and b) May 2012. The symbols are the same as described in Fig. 1

Fig. 7.

Two-dimensional food web structure in stream ecosystems estimated from the stable nitrogen isotope ratios of glutamic acid and phenylalanine. The symbols are the same as described in

Fig. 1; periphyton: n = 13; terrestrial C3 litter: n = 7; grazer: n = 9; shredder: n = 5; filter feeder: n = 8; other invertebrates: n = 4; predator: n = 27; demarsal fish (goby): n = 10; and other fishes: n = 6. The bars indicate standard deviations



79

80 Figure 1

81

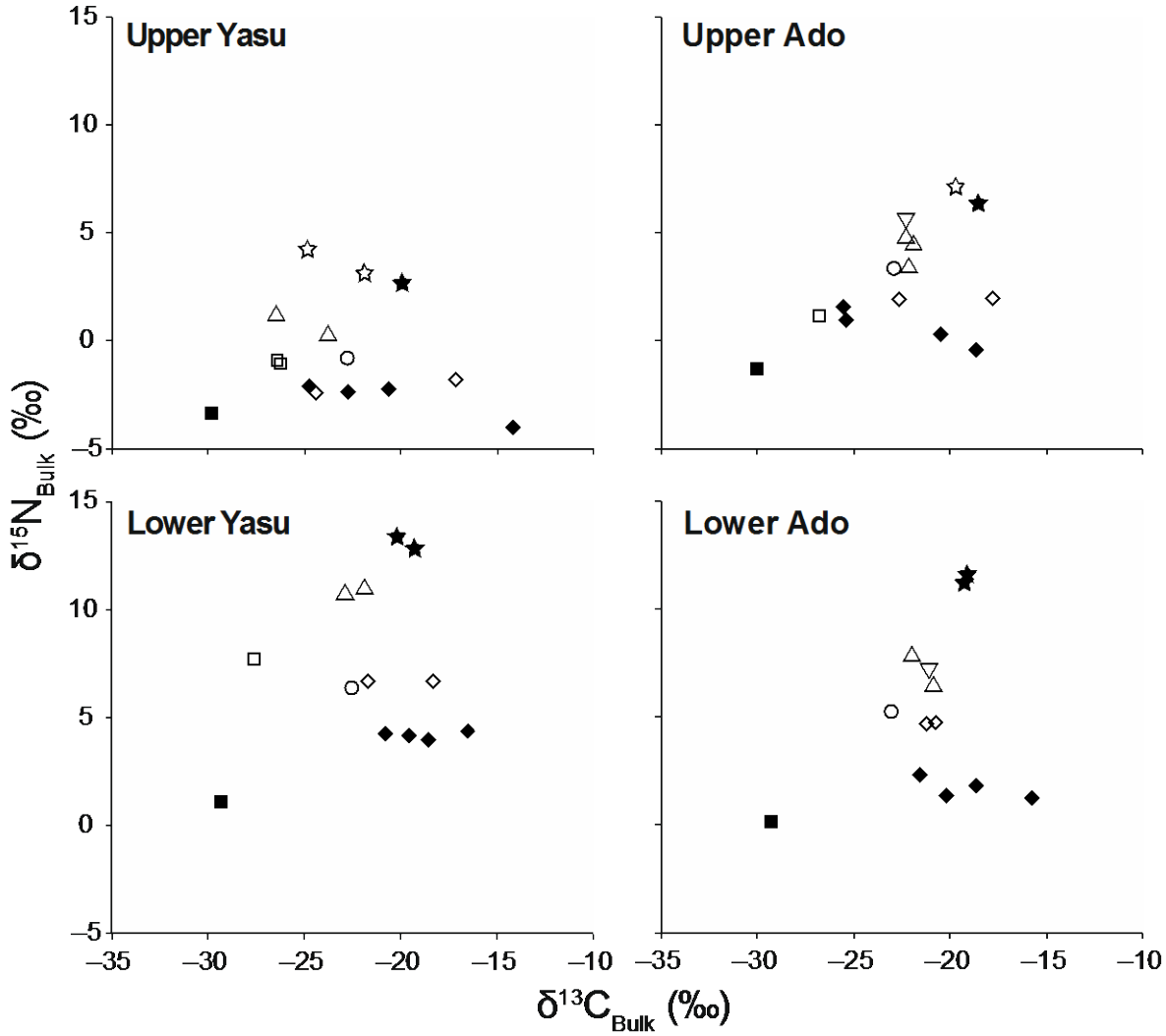
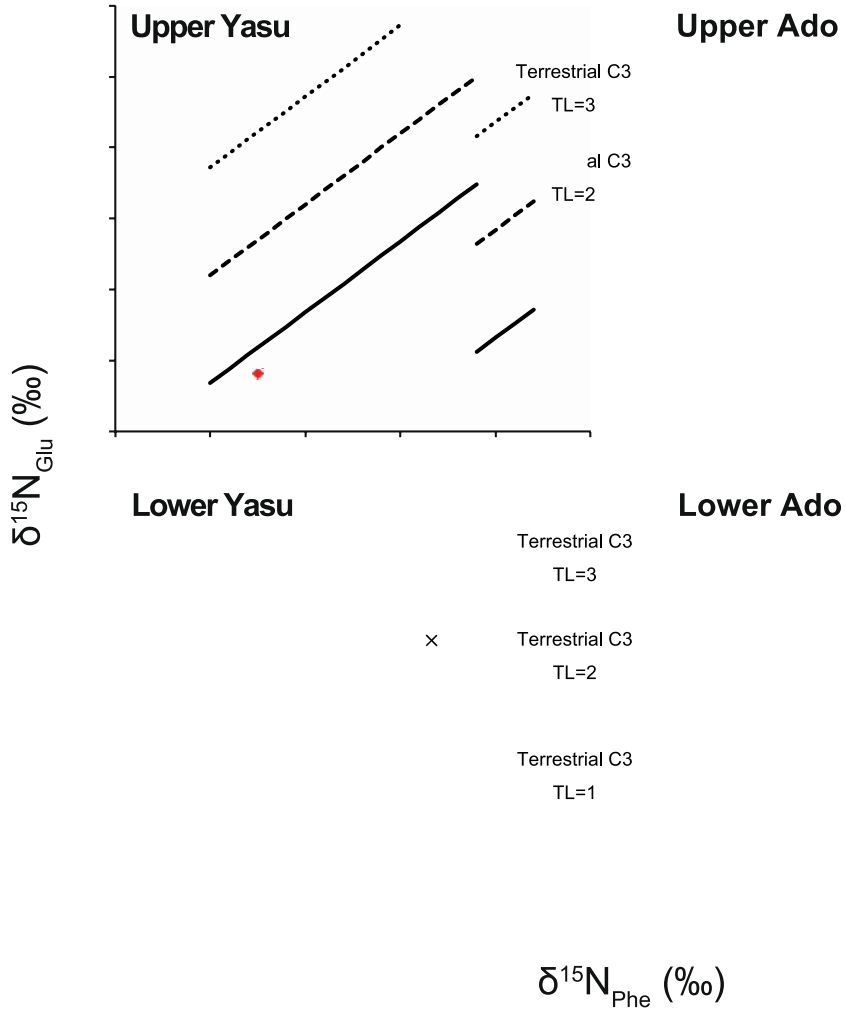

Upper Yasu
Upper Ado
Lower Yasu
Lower Ado
15
10
5
0
−5
−35
−30
−25
−20
−15
−10
δ15NBulk (‰)
δ13CBulk (‰)

Figure 2



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86 Figure 3

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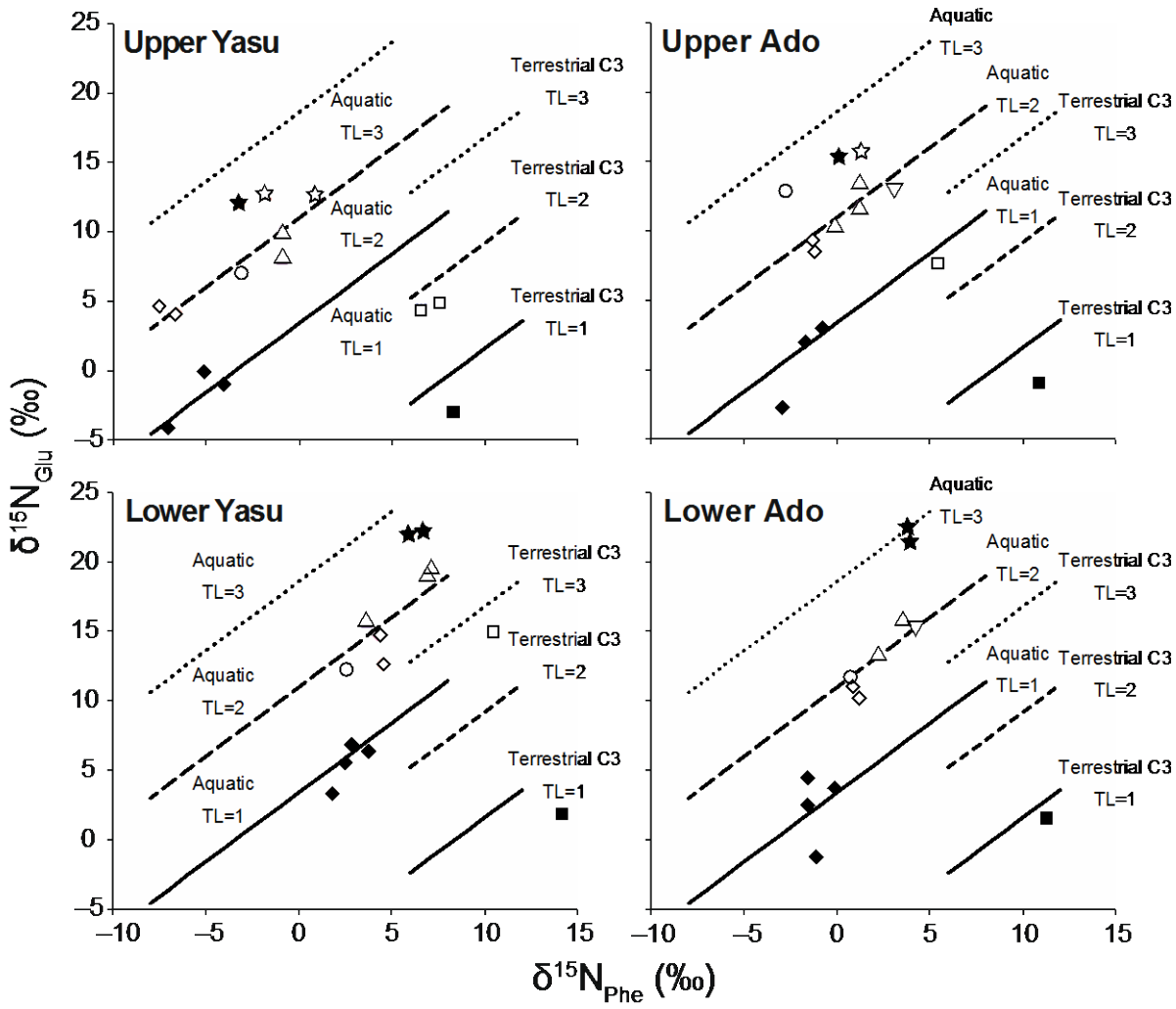

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89 Figure 4

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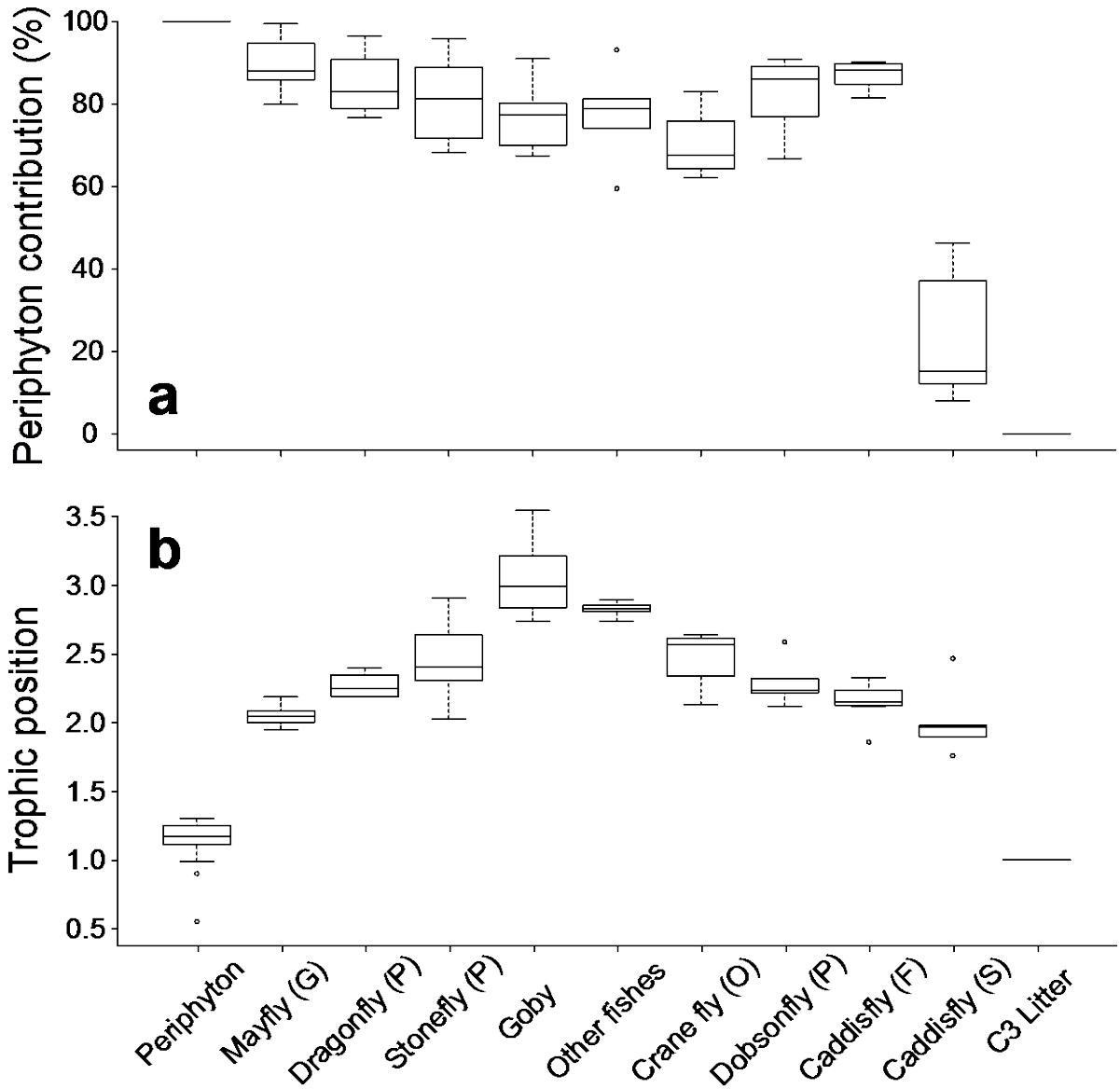

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92 Figure 5

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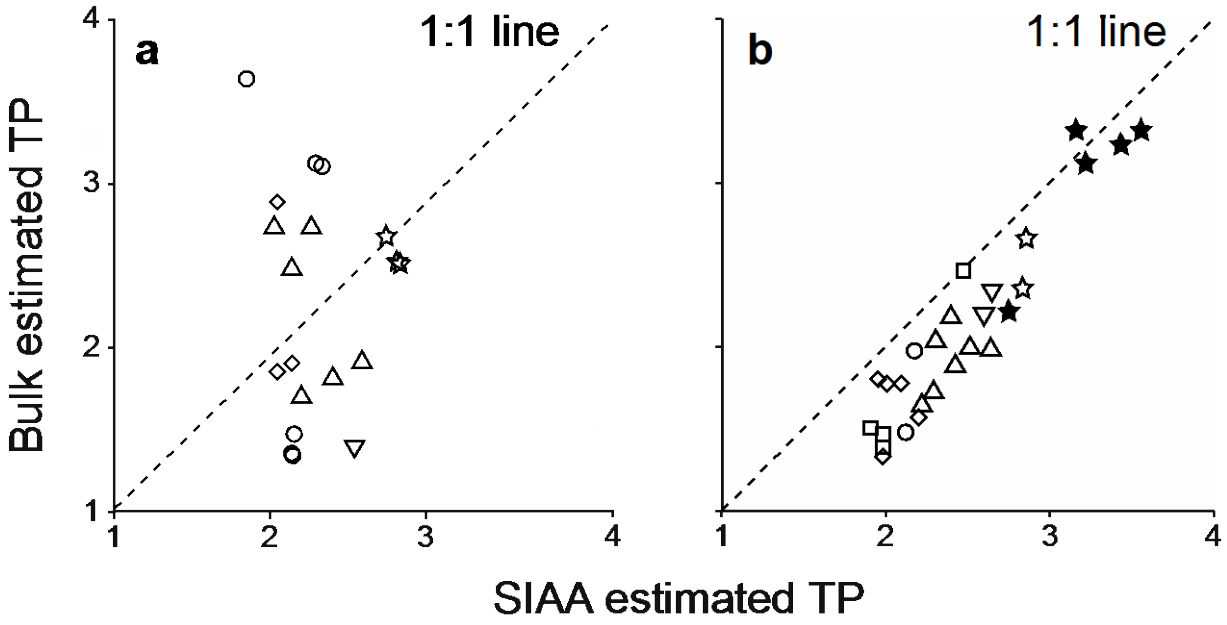

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95 Figure 6

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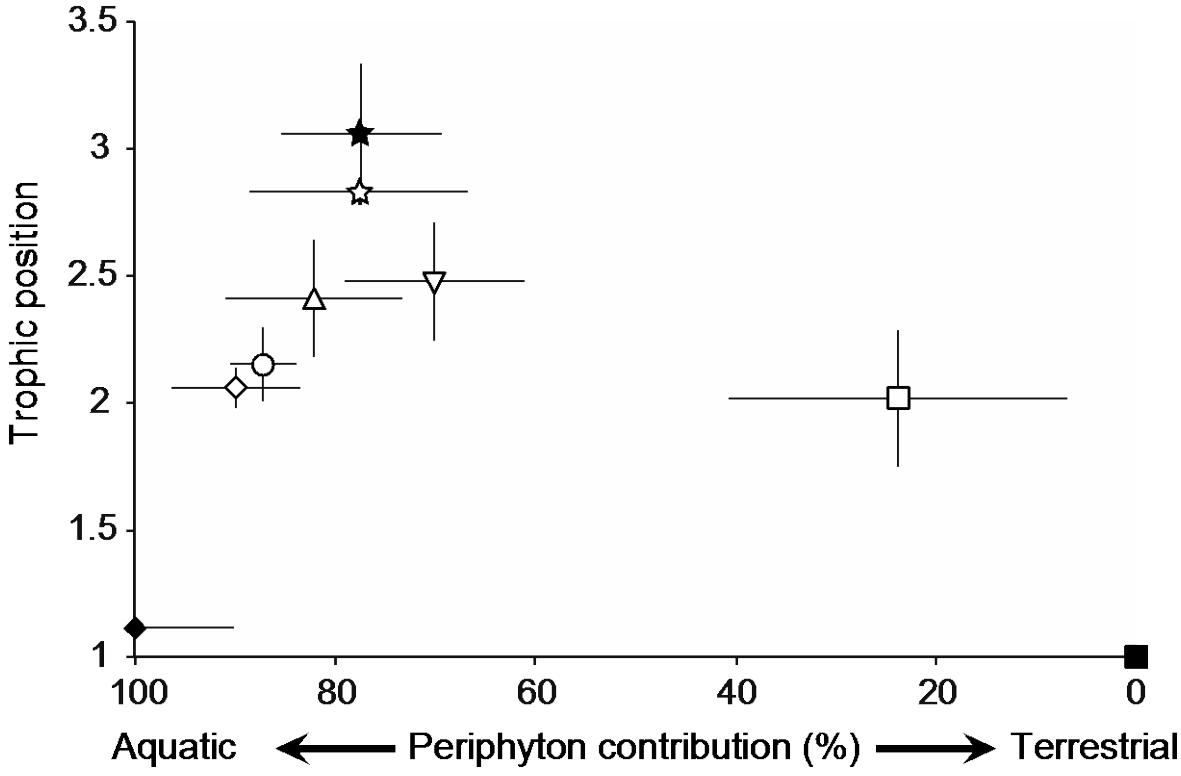
3.5
3
2.5
2
1.5
1
Trophic position
100
80
60
40
20
0
Aquatic
Periphyton contribution (%)
Terrestrial

Figure 7

Stable nitrogen isotopic composition of amino acids reveals food web structure in stream ecosystems

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Running head: Amino acid $\delta^{15}N$ of stream animals

Appendices

Source contribution to an animal (i.e., Eq. 2 and 4) is algebraically induced using two-isotope and two-source mixing model as follows: if X and Y (i.e., $\delta^{15}N_{Bulk}$ and $\delta^{13}C_{Bulk}$ in Eq. 2 and 3; $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ in Eq. 4 and 5) are assimilated by an animal in the same proportions and in the same trophic transfer pathways, then:

$$\delta X[A] = f\{\delta X[P] + \Delta_X (TP - 1)\} + (1 - f)\{\delta X[L] + \Delta_X (TP - 1)\}$$

$$\delta Y[A] = f\{\delta Y[P] + \Delta_Y (TP - 1)\} + (1 - f)\{\delta Y[L] + \Delta_Y (TP - 1)\}$$

where $0 \leq f \leq 1$ and $\delta X[A]$, $\delta Y[A]$, $\delta X[L]$, $\delta Y[L]$, $\delta X[P]$, and $\delta Y[P]$ are δX and δY of animal [A], those of C3 litter [L], and those of periphyton [P] in each site, respectively. Δ_X and Δ_Y are trophic enrichment factors for δX and δY, respectively. TP is trophic position of animal [A]. If both Δ_X and Δ_Y are not zero, and TP is larger than 1, then:

$$f\left(\frac{\delta X[P] - \delta X[L]}{\Delta_X (TP - 1)} - \frac{\delta Y[P] - \delta Y[L]}{\Delta_Y (TP - 1)}\right) = \frac{\delta X[A] - \delta X[L]}{\Delta_X (TP - 1)} - \frac{\delta Y[A] - \delta Y[L]}{\Delta_Y (TP - 1)}$$

Therefore, f is finally represented regardless of TP of animal [A] as:

$$f = \frac{\frac{\delta X[A] - \delta X[L]}{\Delta_X} - \frac{\delta Y[A] - \delta Y[L]}{\Delta_Y}}{\frac{\delta X[P] - \delta X[L]}{\Delta_X} - \frac{\delta Y[P] - \delta Y[L]}{\Delta_Y}}$$

TP of animal [A] (i.e., Eq. 3 and 5) is induced as:

$$\Delta_X \text{ TP} = f\,(\delta X[A] - \delta X[P]) + (1 - f)\,(\delta X[A] - \delta X[L]) + \Delta_X$$

$$\Delta_Y \text{ TP} = f\,(\delta Y[A] - \delta Y[P]) + (1 - f)\,(\delta Y[A] - \delta Y[L]) + \Delta_Y$$

If Δ_X is not equal to Δ_Y, then:

$$\text{TP} = \frac{\delta X[A] - \delta Y[A] - \{f\,(\delta X[P] - \delta Y[P]) + (1 - f)(\delta X[L] - \delta Y[L])\}}{\Delta_X - \Delta_Y} + 1$$

Table A1.

Geographic information of the study sites

	Yasu		Ado	
	Upper	Lower	Upper	Lower
Latitude	35° 00' 05" N	34° 59' 04" N	35° 12' 35" N	35° 21' 00" N
Longitude	136° 23' 31" E	136° 07' 15" E	135° 51' 20" E	136° 00' 02" E
Watershed area (km^2)	4.2	294.7	25.4	298.5
Mean width (m)	8.2	60.8	17.7	31.0
Elevation (m a.s.l.)	508	145	435	108
Canopy cover (%) in November 2011	48.4	13.8	68.1	12.2
Canopy cover (%) in May 2012	58.5	14.0	78.2	19.1
Substrate	Cobble	Cobble	Cobble	Cobble/Sand

Table A2.

Analysis of variance table for periphyton contributions (relative to C3 litter) to animals estimated using a SIAA based two-source mixing model

	Df	Sum Sq	Mean Sq	*F* value	*p* value
Season	1	1135	1135	14.6	<0.001
River	1	2248	2248	28.8	<0.001
Site	1	51	50	0.6	0.424
Animal group	8	14426	1803	23.1	<0.001
Residuals	57	4446	78		

Table A3.

Analysis of variance table for the trophic positions of animals estimated using a SIAA based two-source mixing model

	Df	Sum Sq	Mean Sq	*F* value	*p* value
Season	1	0.33	0.33	8.7	0.005
River	1	0.05	0.05	1.4	0.235
Site	1	0.24	0.24	6.3	0.015
Animal group	8	7.60	0.95	25.2	<0.001
Residuals	57	2.15	0.04		

Table A4.

Full dataset analyzed in this study. N/A: Not available

					SIAA				Bulk			
River	Site	Specimen	Scientific name	FFG	$\delta^{15}N_{Glu}$ (‰)	$\delta^{15}N_{Phe}$ (‰)	Periphyton contribution (%)	Trophic position	$\delta^{15}N_{Bulk}$ (‰)	$\delta^{13}C_{Bulk}$ (‰)	Periphyton contribution (%)	Trophic position
Yasu	Upper	Periphyton			-0.88	-2.51	96.57	0.90	-2.46	-15.51		
Yasu	Upper	Periphyton			-0.17	-2.21	94.60	0.99	-2.42	-15.35		
Yasu	Upper	Periphyton			1.01	-4.03	108.83		-1.55	-20.71		
Ado	Upper	Periphyton			4.63	1.12	82.81	1.28	1.38	-20.49		
Ado	Upper	Periphyton			1.13	-3.16	116.70		0.25	-17.43		
Ado	Upper	Periphyton			4.38	1.65	78.36	1.25	0.54	-18.55		
Ado	Upper	Periphyton			0.65	-3.48	119.07		-0.24	-14.55		
Ado	Upper	Periphyton			1.07	-1.51	103.07		0.20	-17.61		
Yasu	Lower	Periphyton			8.73	6.60			6.89	-18.35		
Yasu	Lower	Periphyton			13.23	9.08			8.44	-24.02		
Yasu	Lower	Periphyton			10.61	4.89			6.73	-19.53		
Yasu	Lower	Periphyton			12.47	6.62			8.52	-22.42		
Ado	Lower	Periphyton			6.45	1.25	84.47	1.27	2.39	-23.48		
Ado	Lower	Periphyton			3.39	-1.35	104.13		1.71	-20.86		
Ado	Lower	Periphyton			5.20	-2.12	111.00		1.21	-20.77		
Ado	Lower	Periphyton			4.32	-0.84	100.40		1.81	-18.86		
Yasu	Upper	Periphyton			-1.02	-4.08	90.44	1.11	-2.32	-22.77		
Yasu	Upper	Periphyton			-4.12	-7.08	111.08		-4.03	-14.19		
Yasu	Upper	Periphyton							-2.21	-20.62		
Yasu	Upper	Periphyton			-0.11	-5.15	98.48	1.21	-2.13	-24.80		
Ado	Upper	Periphyton			3.06	-0.84	93.10	1.30	0.99	-25.37		

Table A4 (continued).

						SIAA				Bulk			
Season	River	Site	Specimen	Scientific name	FFG	$\delta^{15}N_{Glu}$ (‰)	$\delta^{15}N_{Phe}$ (‰)	Periphyton contribution (%)	Trophic position	$\delta^{15}N_{Bulk}$ (‰)	$\delta^{13}C_{Bulk}$ (‰)	Periphyton contribution (%)	Trophic position
May	Ado	Upper	Periphyton			2.03	-1.72	99.63	1.16	0.30	-20.46		
May	Ado	Upper	Periphyton							1.57	-25.55		
May	Ado	Upper	Periphyton			-2.75	-2.94	107.27		-0.36	-18.62		
May	Yasu	Lower	Periphyton			6.34	3.77	91.20	1.14	4.37	-16.55		
May	Yasu	Lower	Periphyton			3.35	1.78	107.12		3.97	-18.56		
May	Yasu	Lower	Periphyton			6.80	2.84	99.42	1.17	4.24	-20.81		
May	Yasu	Lower	Periphyton			5.49	2.45	102.26		4.19	-19.55		
May	Ado	Lower	Periphyton			2.44	-1.56	105.62		1.27	-15.79		
May	Ado	Lower	Periphyton			3.77	-0.13	92.75	1.18	2.35	-21.60		
May	Ado	Lower	Periphyton			4.49	-1.53	104.38		1.38	-20.20		
May	Ado	Lower	Periphyton			-1.19	-1.16	99.07	0.55	1.83	-18.69		
November	Yasu	Upper	Mayfly	Heptageniidae spp.	Grazer	6.14	-3.76	108.75		4.66	-23.02	38.94	3.03
November	Ado	Upper	Mayfly	Heptageniidae spp.	Grazer	11.59	1.10	85.82	2.15	4.40	-17.67	103.93	
November	Ado	Upper	Mayfly	Heptageniidae spp.	Grazer	10.82	1.78	79.93	2.05	3.90	-20.65	78.77	2.00
November	Yasu	Lower	Mayfly	Heptageniidae spp.	Grazer	17.35	8.02			2.99	-21.18	100.00	
November	Yasu	Lower	Mayfly	Heptageniidae spp.	Grazer	17.75	8.04			3.68	-21.36	100.00	
November	Ado	Lower	Mayfly	*Baetis* spp.	Grazer	12.98	0.29	94.76	2.04	9.76	-20.78	94.23	3.31
May	Yasu	Upper	Mayfly	Heptageniidae spp.	Grazer	4.64	-7.52	117.47		-2.42	-24.44	45.87	1.25
May	Yasu	Upper	Mayfly	Heptageniidae spp.	Grazer	4.09	-6.62	110.72		-1.84	-17.18	108.67	
May	Ado	Upper	Mayfly	Heptageniidae spp.	Grazer	8.55	-1.21	98.19	1.98	1.98	-22.64	99.43	1.40
May	Ado	Upper	Mayfly	Heptageniidae spp.	Grazer	9.37	-1.34	99.47	2.07	1.99	-17.76	172.62	

Table A4 (continued).

River	Site	Specimen	Scientific name	FFG	SIAA				Bulk			
					$\delta^{15}N_{Glu}$ (‰)	$\delta^{15}N_{Phe}$ (‰)	Periphyton contribution (%)	Trophic position	$\delta^{15}N_{Bulk}$ (‰)	$\delta^{13}C_{Bulk}$ (‰)	Periphyton contribution (%)	Trophic position
Yasu	Lower	Mayfly	Heptageniidae spp.	Grazer	12.61	4.54	87.26	1.95	6.65	-21.77	56.64	2.12
Yasu	Lower	Mayfly	*Baetis* spp.	Grazer	14.69	4.28	90.43	2.19	6.71	-18.34	87.50	1.86
Ado	Lower	Mayfly	Heptageniidae spp.	Grazer	10.25	1.17	84.85	2.00	4.73	-21.20	68.64	2.01
Ado	Lower	Mayfly	Heptageniidae spp.	Grazer	10.99	0.83	87.94	2.09	4.76	-20.82	72.29	2.00
Yasu	Upper	Dragon fly	Gomphidae spp.	Predator	9.30	0.63	76.64	2.20	1.06	-24.37	35.59	1.96
Ado	Upper	Dragon fly	Gomphidae spp.	Predator	13.61	1.31	84.93	2.40	3.83	-19.91	85.42	1.95
Yasu	Lower	Dragon fly	Gomphidae spp.	Predator	15.63	4.50			7.00	-20.89	100.00	
Ado	Lower	Dragon fly	Gomphidae spp.	Predator	13.64	2.01	81.24	2.19	4.18	-18.15	148.69	
Yasu	Lower	Dragon fly	Gomphidae spp.	Predator	15.79	3.62	96.58	2.30	7.48	-22.42	48.94	2.43
Yasu	Upper	Stonefly	*Kamimuria tibialis*	Predator	10.44	-2.45	100.44		-0.65	-20.52	63.65	1.49
Yasu	Upper	Stonefly	Chloroperlidae spp.	Predator	8.98	-0.90	88.14	2.14	4.47	-17.80	73.61	3.01
Ado	Upper	Stonefly	*Kamimuria tibialis*	Predator	13.48	1.03	87.18	2.38	0.11	-20.88	84.59	0.86
Ado	Upper	Stonefly	*Kamimuria tibialis*	Predator	14.50	2.13	78.52	2.51				
Ado	Upper	Stonefly	*Kamimuria tibialis*	Predator	13.45	1.77	81.11	2.38				
Ado	Upper	Stonefly	*Kamimuria tibialis*	Predator	14.82	1.56	83.36	2.55				
Ado	Upper	Stonefly	*Kamimuria tibialis*	Predator	13.28	0.81	88.98	2.36				
Ado	Upper	Stonefly	*Kamimuria tibialis*	Predator	12.88	0.23	93.57	2.31				
Ado	Upper	Stonefly	*Paragnetina tinctipennis*	Predator	17.34	0.18	95.80	2.87				
Yasu	Lower	Stonefly	*Kamimuria tibialis*	Predator	20.51	7.84			3.99	-17.61	100.00	
Yasu	Lower	Stonefly	*Kamimuria tibialis*	Predator	19.78	7.50			1.24	-22.37	100.00	
Yasu	Lower	Amphipods	*Gammarus nipponensis*	Predator	20.63	6.90						

Table A4 (continued).

River	Site	Specimen	Scientific name	FFG	SIAA				Bulk			
					$\delta^{15}N_{Glu}$ (‰)	$\delta^{15}N_{Phe}$ (‰)	Periphyton contribution (%)	Trophic position	$\delta^{15}N_{Bulk}$ (‰)	$\delta^{13}C_{Bulk}$ (‰)	Periphyton contribution (%)	Trophic position
Ado	Lower	Stonefly	*Kamimuria tibialis*	Predator	12.71	0.92	89.60	2.03	8.85	-22.30	76.17	3.16
Ado	Lower	Stonefly	*Kamimuria tibialis*	Predator	14.63	0.80	91.32	2.26	9.05	-21.21	90.47	3.13
Ado	Lower	Stonefly	*Oyamia lugubris*	Predator	11.46	-0.51	100.61					
Yasu	Upper	Stonefly	*Niponiella limbatella*	Predator	8.17	-0.92	70.80	2.29	0.31	-23.85	45.48	2.05
Yasu	Upper	Stonefly	*Niponiella limbatella*	Predator	9.98	-0.95	71.65	2.51	1.29	-26.50	20.07	2.35
Ado	Upper	Stonefly	*Niponiella limbatella*	Predator	11.82	1.19	80.61	2.42	4.52	-21.98	100.38	
Ado	Upper	Stonefly	*Niponiella limbatella*	Predator	13.55	1.19	81.28	2.64	4.84	-22.34	93.88	2.27
Yasu	Lower	Stonefly	*Niponiella limbatella*	Predator	19.20	6.86	70.14	2.85	10.82	-22.88	37.72	3.51
Yasu	Lower	Stonefly	*Niponiella limbatella*	Predator	19.62	7.09	68.32	2.91	10.98	-21.95	45.81	3.49
Ado	Lower	Stonefly	Chloroperlidae spp.	Predator	13.36	2.21	77.71	2.39	6.51	-20.93	67.12	2.54
Ado	Lower	Stonefly	Chloroperlidae spp.	Predator	15.86	3.50	68.23	2.72	7.92	-22.04	53.03	3.03
Yasu	Lower	Fish (Goby)	*Rhinogobius kurodai*		21.79	5.88			13.12			
Yasu	Lower	Fish (Goby)	*Rhinogobius kurodai*		19.86	5.06			11.22	-18.95	100.00	
Ado	Lower	Fish (Goby)	*Rhinogobius kurodai*		20.21	2.79	77.58	3.03				
Ado	Lower	Fish (Goby)	*Rhinogobius kurodai*		19.07	2.79	77.18	2.88	9.31	-17.51	140.86	
Ado	Lower	Fish (Goby)	*Rhinogobius kurodai*		18.09	2.37	80.10	2.75	9.22	-16.32	157.72	
Ado	Lower	Fish (Goby)	*Rhinogobius kurodai*		19.44	3.71	69.92	2.96	9.86	-17.33	141.57	
Yasu	Upper	Fish (Goby)	*Rhinogobius flumineus*		12.03	-3.26	89.16	2.74	2.69	-19.95	74.84	2.74
Ado	Upper	Fish (Goby)	*Rhinogobius flumineus*		15.38	0.05	90.95	2.84	6.42	-18.59	144.51	
Yasu	Lower	Fish (Goby)	*Rhinogobius kurodai*		22.32	6.66	73.19	3.22	12.82	-19.32	65.64	3.85
Yasu	Lower	Fish (Goby)	*Rhinogobius kurodai*		21.97	5.94	79.25	3.15	13.39	-20.19	56.59	4.10

Table A4 (continued).

River	Site	Specimen	Scientific name	FFG	SIAA				Bulk			
					$\delta^{15}N_{Glu}$ (‰)	$\delta^{15}N_{Phe}$ (‰)	Periphyton contribution (%)	Trophic position	$\delta^{15}N_{Bulk}$ (‰)	$\delta^{13}C_{Bulk}$ (‰)	Periphyton contribution (%)	Trophic position
Ado	Lower	Fish (Goby)	*Rhinogobius kurodai*		21.57	3.89	67.44	3.43	11.25	-19.26	72.60	3.91
Ado	Lower	Fish (Goby)	*Rhinogobius kurodai*		22.54	3.75	68.95	3.55	11.63	-19.12	73.09	4.02
Yasu	Upper	Fish (Trout)	*Oncorhynchus masou ishikawae*		14.18	1.21	74.13	2.81	4.68	-22.65	41.35	3.04
Ado	Upper	Fish (Trout)	*Oncorhynchus masou ishikawae*		17.13	0.49	93.13	2.84	6.89	-18.58	90.78	2.83
Ado	Upper	Fish (Minnow)	*Rhynchocypris* sp.		16.35	2.19	78.85	2.74	7.26	-23.06	50.66	3.12
Yasu	Lower	Fish (Chub)	*Nipponocypris temminckii*		22.08	6.56			12.27	-22.25	100.00	
Yasu	Upper	Fish (Trout)	*Oncorhynchus masou ishikawae*		12.65	-1.82	78.93	2.83	3.11	-21.93	56.57	2.87
Yasu	Upper	Fish (Minnow)	*Rhynchocypris oxycephalus jouyi*		12.58	0.86	59.50	2.86	4.23	-24.86	28.37	3.21
Ado	Upper	Fish (Minnow)	*Rhynchocypris oxycephalus jouyi*		15.64	1.30	81.29	2.90	7.09	-19.77	124.56	
Yasu	Upper	Crane fly	Tipulidae spp.	Other invertebrates	12.03	1.81	68.73	2.55	-0.35	-21.43	57.22	1.57
Ado	Lower	Crane fly	Tipulidae spp.	Other invertebrates	13.27	1.78	82.94	2.13	-1.28	-18.43	162.64	
Ado	Upper	Crane fly	Tipulidae spp.	Other invertebrates	12.94	3.06	66.41	2.59	5.65	-22.29	91.77	2.52
Ado	Lower	Crane fly	Tipulidae spp.	Other invertebrates	15.22	4.22	62.19	2.64	7.14	-21.08	64.20	2.74
Ado	Upper	Dobson fly	*Protohermes grandis*	Predator	15.12	3.59	66.78	2.59	4.18	-20.84	76.50	2.09
Ado	Upper	Dobson fly	*Protohermes grandis*	Predator	12.37	2.22	76.93	2.24				
Ado	Upper	Dobson fly	*Protohermes grandis*	Predator	12.97	1.14	86.10	2.32				
Ado	Upper	Dobson fly	*Protohermes grandis*	Predator	11.39	0.70	89.03	2.12				
Ado	Upper	Dobson fly	*Protohermes grandis*	Predator	10.37	-0.18	90.82	2.22	3.46	-22.15	101.56	
Yasu	Upper	Caddisfly	Hydropsychidae spp.	Filter feeder	6.72	-1.28	90.17	1.86	9.63	-26.50	8.28	4.46
Ado	Upper	Caddisfly	Hydropsychidae spp.	Filter feeder	12.79	0.66	89.98	2.30	9.12	-23.62	41.93	3.71
Ado	Upper	Caddisfly	Hydropsychidae spp.	Filter feeder	13.07	0.72	89.61	2.33	9.22	-21.93	56.50	3.67

Table A4 (continued).

River	Site	Specimen	Scientific name	FFG	SIAA				Bulk			
					$\delta^{15}N_{Glu}$ (‰)	$\delta^{15}N_{Phe}$ (‰)	Periphyton contribution (%)	Trophic position	$\delta^{15}N_{Bulk}$ (‰)	$\delta^{13}C_{Bulk}$ (‰)	Periphyton contribution (%)	Trophic position
Yasu	Lower	Caddisfly	*Stenopsyche marmorata*	Filter feeder	18.85	8.26			3.46	-17.48	100.00	
Yasu	Lower	Caddisfly	*Stenopsyche marmorata*	Filter feeder	19.95	7.66			3.55	-21.31	100.00	
Ado	Lower	Caddisfly	Hydropsychidae spp.	Filter feeder	13.47	1.24	87.32	2.14	3.05	-21.79	102.07	
Ado	Lower	Caddisfly	Hydropsychidae spp.	Filter feeder	13.18	1.96	81.45	2.13	2.97	-22.59	91.21	1.33
Ado	Lower	Caddisfly	*Stenopsyche marmorata*	Filter feeder	13.48	1.76	83.18	2.16	3.36	-22.95	85.06	1.49
Yasu	Upper	Caddisfly	Hydropsychidae spp.	Filter feeder	7.05	-3.11	86.31	2.12	-0.78	-22.81	56.87	1.73
Ado	Upper	Caddisfly	Hydropsychidae spp.	Filter feeder	12.82	-2.78	112.12		3.36	-22.93	90.24	1.85
Yasu	Lower	Caddisfly	Hydropsychidae spp.	Filter feeder	12.23	2.51	104.64		6.38	-22.62	49.52	2.10
Ado	Lower	Caddisfly	Hydropsychidae spp.	Filter feeder	11.70	0.73	89.03	2.18	5.26	-23.05	49.22	2.26
Yasu	Upper	Caddisfly	*Goerodes* spp.	Shredder	5.06	8.91	12.22	1.76				
Yasu	Upper	Caddisfly	*Goerodes* spp.	Shredder	4.37	6.54	15.26	1.90	-0.86	-26.46	24.89	1.72
Yasu	Upper	Caddisfly	*Goerodes* spp.	Shredder	4.87	7.55	8.06	1.98	-1.07	-26.27	26.98	1.66
Ado	Upper	Caddisfly	*Goerodes* spp.	Shredder	7.68	5.36	46.35	1.97	1.19	-26.81	39.72	1.50
Yasu	Lower	Caddisfly	*Goerodes* spp.	Shredder	14.97	10.48	37.05	2.47	7.66	-27.63	1.50	2.91
Yasu	Upper	C3 Litter			-1.15	10.21	0.00	1.00	-2.10	-30.51		
Ado	Upper	C3 Litter			2.56	11.06	0.00	1.00	-0.72	-30.71		
Ado	Lower	C3 Litter			1.14	11.50	0.00	1.00	-0.26	-29.94		
Yasu	Upper	C3 Litter			-3.04	8.27	0.00	1.00	-3.35	-29.86		
Ado	Upper	C3 Litter			-0.90	10.84	0.00	1.00	-1.26	-30.03		
Yasu	Lower	C3 Litter			1.88	14.11	0.00	1.00	1.13	-29.33		
Ado	Lower	C3 Litter			1.59	11.22	0.00	1.00	0.15	-29.27		
Yasu	Lower	POM			14.03	6.66			7.21	-24.96		

Figure legends

Fig. A1.

Study sites draining the Lake Biwa basin, central Japan. Areas surrounded by lines indicate watersheds of the main stems of the Yasu and Ado rivers. Open and solid stars in the Yasu and Ado rivers indicate the upper and lower sites studied, respectively

Fig. A2.

Landscapes of the study sites

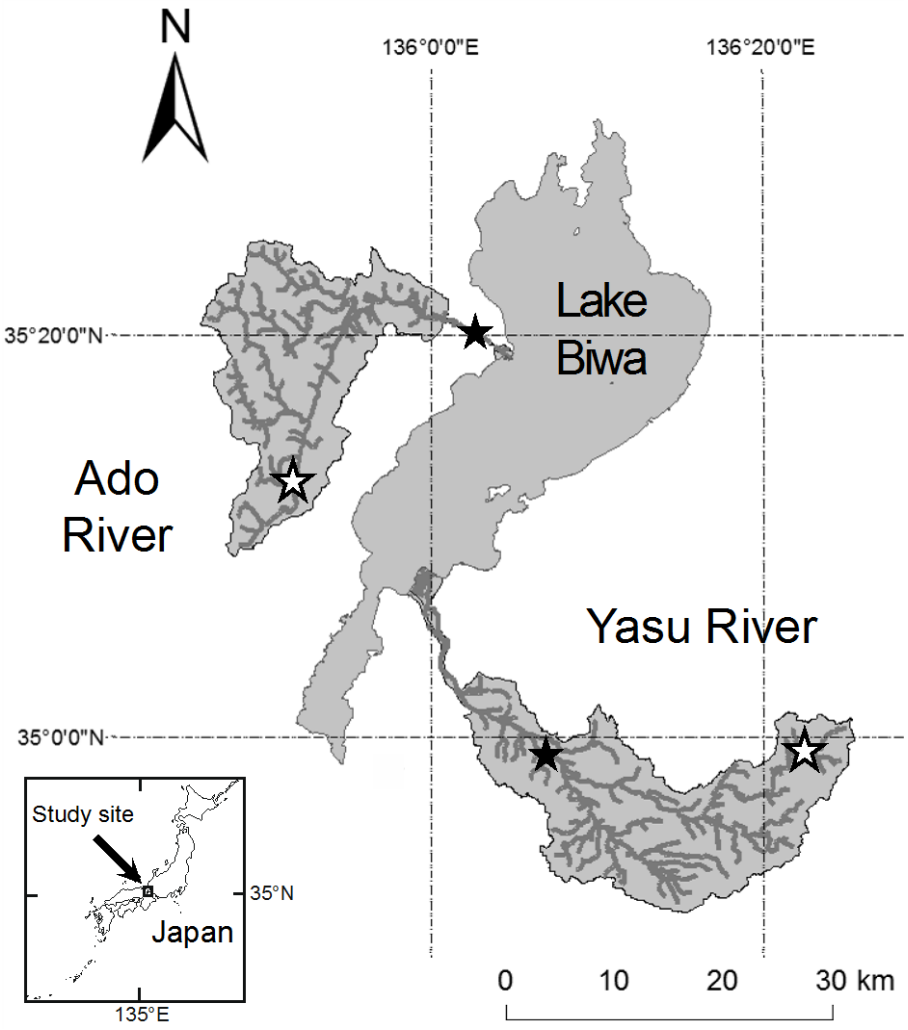
N
136°0'0"E
136°20'0"E
35°20'0"N
35°0'0"N
Lake
Biwa
Ado
River
Yasu River
Study site
35°N
Japan
135°E
0
10
20
30 km

Figure A1

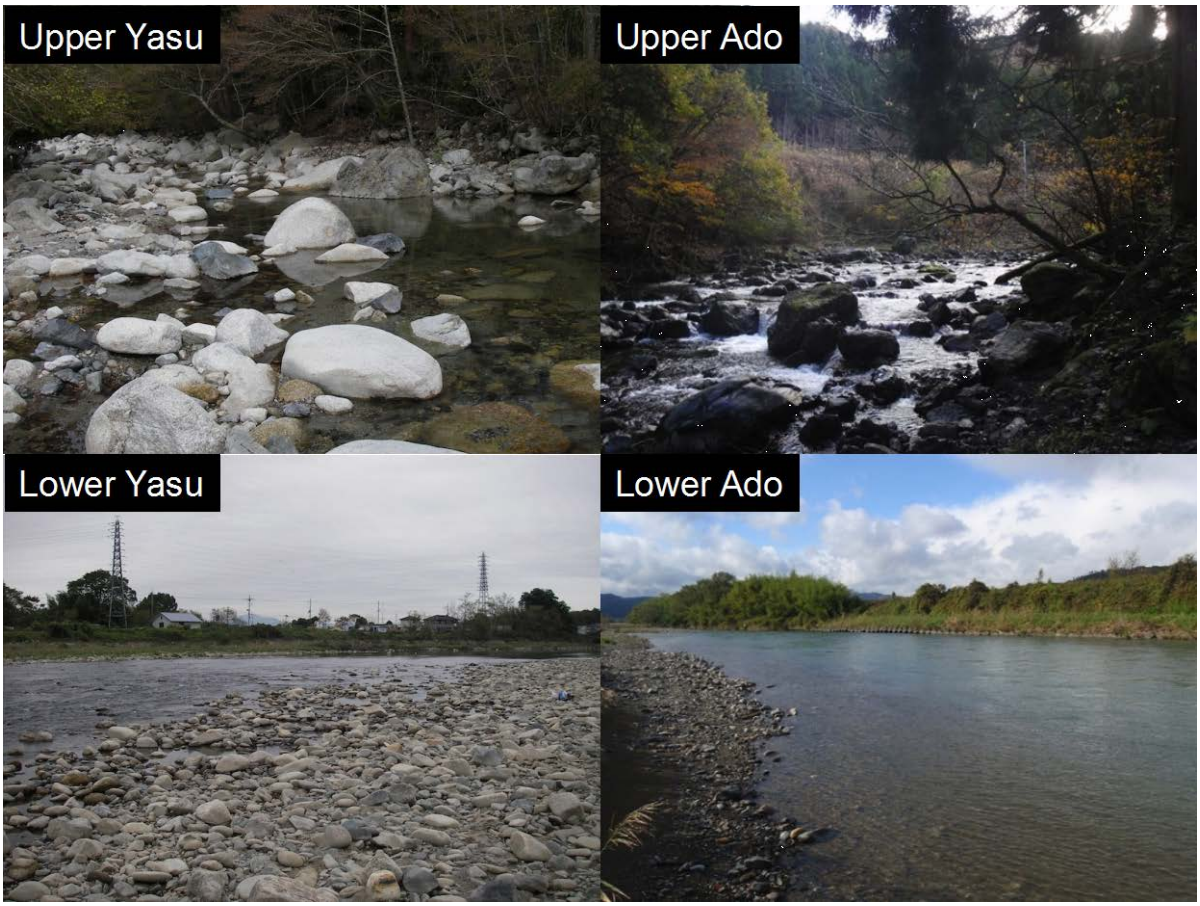

Figure A2