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Carbon and nitrogen stable isotope ratios of macroinvertebrates in the littoral zone of Lake Biwa as indicators of anthropogenic activities in the watershed

Abstract Carbon and nitrogen stable isotope ratios (δ^{13}C and δ^{15}N) of macroinvertebrates inhabiting littoral zones of lakes can serve as useful indicators of material loading from the watershed. We collected snails (*Semisulcospira* spp.) and bivalves (*Unio douglasiae biwae* Kobelt) from 29 littoral sites in Lake Biwa near the mouths of river tributaries with various human population density (HPD) and land-use patterns. The δ^{13}C and δ^{15}N signatures were determined for three potential food sources: particulate organic matter in the pelagic zone (PPOM), riverine particulate organic matter from tributaries (RPOM) and epilithic organic matter in the littoral zone (EOM). The stable isotope mixing model revealed that snails relied mainly on EOM, and bivalves on PPOM and RPOM. Multiple regression analysis showed that intersite variation in δ^{15}N for snails was best explained by HPD, while variation in δ^{15}N of EOM and nitrate was explained to a lesser extent by HPD. Comparison with isotope signatures of their sources and riverine nutrients revealed that snails assimilated anthropogenic nitrogen from wastewater in the watershed. Our results also showed that the δ^{13}C value of bivalves was marginally related to the fraction of paddy fields in the watersheds. In conclusion, the isotope signatures of macroinvertebrates inhabiting the littoral zone can be useful indicators of anthropogenic impacts from the watershed.

Keywords Material loading · Lake Biwa · Eutrophication · Stable isotope · Benthos

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Introduction

Lake Biwa is the largest freshwater lake in Japan and is an important water resource for the 14 million people living in its watershed and surrounding area. Lake Biwa consists of a north basin and a much smaller and shallower south basin, which has been more severely eutrophicated than the north basin (Shiga Prefectural Government 2008). The lake has a total surface area of 674 km^2, a volume of 27.5 km^3, and maximum depth of 104 m (Okuda and Kumagai 1995), 118 tributary rivers (Shiga Prefectural Government 2009), and only one outflow river at the south end. The water quality and biodiversity of Lake Biwa have been deteriorating since the 1960s, when there were drastic changes in human activities in the watershed, including an increase in population, alterations of human life and agricultural styles, industrialization, and exploitation of the littoral zone (Nakanishi and Sekino 1996). Residential, agricultural, and industrial wastewater discharges in the watershed are recognized as causes of water quality deterioration in Lake Biwa. For example, agricultural wastewater from paddy fields has high levels of suspended solids that are rich in nitrogen and phosphorous and greatly impact the water quality, especially during the paddy flooding season from late April to early May (Ohkubo and Azuma 2005). Residential wastewater

contributes to the loading to Lake Biwa, accounting for 24% of the total nitrogen loading and 34% of the total phosphorus loading per day (Shiga Prefectural Government 2008). As it is difficult to determine how and to what extent each tributary contributes to the material loading of the lake as a whole, establishment of a reliable and convenient indicator of loading derived from human activities in watersheds is urgently needed.

Stable isotope analysis is a promising technique for analyzing the sources of material loading in watersheds. Nitrogen isotope ratio ($\delta^{15}N$) has mainly been used to quantify the trophic level of a consumer since animals have $\delta^{15}N$ values that are increased by 3.4‰ on average relative to the food source (Minagawa and Wada 1984; Vander Zanden and Rasmussen 2001; Post 2002). Further, nitrogen loading in watersheds of several lakes and ponds has been examined by using the $\delta^{15}N$ value of consumers (Cabana and Rasmussen 1996; Lake et al. 2001; McKinney et al. 2002; Vander Zanden et al. 2005). When primary producers assimilate inorganic nitrogen derived from sewage and agricultural wastewater, which usually contain more ^{15}N than natural nitrogen sources, the $\delta^{15}N$ values of the primary producers and consumers are increased (McClelland et al. 1997; McClelland and Valiela 1998; Kohzu et al. 2008). Thus, the isotopic signals of anthropogenic nitrogen are transferred to consumers through trophic relationships (Cabana and Rasmussen 1996; McKinney et al. 2002). However, few studies have focused on the various types of littoral zones within a lake to elucidate the influence of anthropogenic activities in the watershed (e.g., McKinney et al. 1999).

Carbon isotope ratio ($\delta^{13}C$) has been used to trace food sources of consumers in a food web based on there being little isotopic fractionation of ^{13}C associated with trophic transfer of organic carbon (DeNiro and Epstein 1978; Vander Zanden and Rasmussen 2001; Post 2002). In aquatic ecosystems, the major primary producers are phytoplankton and benthic algae, and benthic algae in the littoral zone tend to have higher $\delta^{13}C$ value than phytoplankton predominating in the pelagic zone, due to the CO_2 boundary layer effect, which limits the diffusion of CO_2 to benthic algal cells (France 1995a). Consequently, consumers in different zones of the lake (littoral vs. pelagic) have different $\delta^{13}C$ values (France 1995b). Another factor contributing to the isotopic variation is contributions from terrestrial C3 plants in allochthonous input, as the $\delta^{13}C$ value is about −28‰ and often different from that of phytoplankton and benthic algae (Peterson and Fry 1987). Based on the differences in $\delta^{13}C$ of the primary producers, it is possible to estimate the relative contributions of allochthonous and autochthonous organic matter to aquatic consumers (e.g., France 1996).

Thus, stable isotope analysis is a powerful tool for identifying food sources of consumers in complex lake food webs (Yoshii 1999), especially within littoral food webs, in which there exist three major food sources: benthic algae, phytoplankton, and terrestrial-derived organic matter. However, in general, the major primary producers in aquatic ecosystems, phytoplankton and benthic algae, are short-lived and have large temporal variations in $\delta^{13}C$ and $\delta^{15}N$ (Cabana and Rasmussen 1996). By contrast, long-lived primary consumers integrate temporal variation in isotope signatures of the primary producers over much longer periods (Cabana and Rasmussen 1996; Post 2002). Among the primary consumers, benthic macroinvertebrates are particularly well-suited to such analyses as they are sedentary and long-lived. They also have specialized feeding habits, such as grazing, shredding, and filter feeding (Thorp and Covich 1991). Therefore, the isotope signatures of benthic macroinvertebrates are more useful than those of primary producers themselves as indicators of food web base in aquatic ecosystems (e.g., Cabana and Rasmussen 1996; McKinney et al. 1999; Vander Zanden and Rasmussen 1999; Post 2002).

The goal of this study was to verify the utility of the $\delta^{13}C$ and $\delta^{15}N$ signatures of macroinvertebrates inhabiting the littoral zone of Lake Biwa as indicators of material loading. We examined how these isotope signatures reflect the impact of local anthropogenic sources through tributary rivers on this lake ecosystem. We used two gastropod groups common to the littoral zone of Lake Biwa: snails (*Semisulcospira* spp.) which feed on benthic algae, and bivalves (*Unio douglasiae biwae* Kobelt) which feed on suspended particulate organic matter.

Methods

Study sites

Field sampling was performed at 32 sites in the north and south basins of Lake Biwa, Shiga Prefecture, Japan (Fig. 1) in November 2005. The mean water depth and the volume of each basin are 44 m and 27.3 km³, respectively, for the north basin, and 3.5 m and 0.2 km³, respectively, for the south basin (Okuda and Kumagai 1995). Samples for stable isotope analysis were collected at littoral sites within 100 m from the mouths of 29 tributary rivers (hereafter, tributary sites). The watersheds of these tributaries differ in human population density (HPD) and land-use pattern (see Table 4 in Appendix). For reference sites, we selected two rock reef sites located at least 1.5 km from the nearest river tributary, as these are expected to not be affected by loading from the watershed (rock reef sites; Nos. 30 and 31 in Fig. 1).

Sample collection and stable isotope analysis

We collected two macroinvertebrate groups classified according to Thorp and Covich (1991): snails (*Semisulcospira* spp.), which were classified as grazers, and bivalves (*Unio douglasiae biwae* Kobelt), which were classified as filter feeders. We considered epilithic
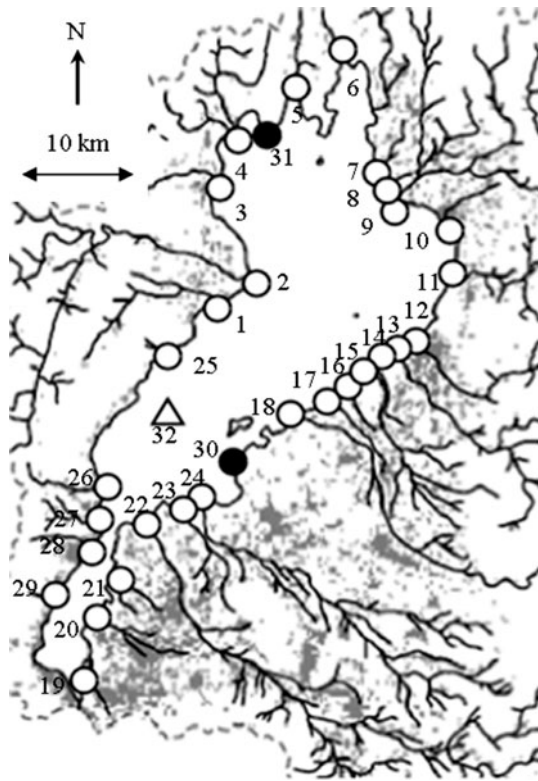
Fig. 1 Map of Lake Biwa showing the sampling sites near the mouths of tributary rivers (*open circles*), sampling sites away from tributaries (*closed circles*) in the littoral zone, and a sampling site in the pelagic zone (*open triangle*). *Numbers* adjacent to the sites correspond to the sampling site numbers

organic matter (EOM), pelagic particulate organic matter (PPOM), and riverine particulate organic matter (RPOM) as potential food sources for these species. EOM samples were collected by scraping the epilithon from five rocks at each littoral sampling site into a plastic container filled with clean water. For PPOM, we collected surface lake water 3.5 km offshore of Ohmi-maiko (35°12′58″N, 135°59′55″E; depth, 70 m; named Ie-1, Mori et al. 1967; No. 32 in Fig. 1) and used the isotope signatures as being representative of those throughout the lake. Briefly, the particulate organic matter represents a mixture of the three food sources due to the re-suspension of sediments. Based on the depth (70 m) and location (approximately 3.5 km from the shoreline) of this pelagic sampling site, we considered the surface sample to be sufficiently unaffected by watershed contributions and by re-suspension of sediment. As the mean depth of the south basin is shallow (3.5 m depth), by definition, there is no pelagic zone in the south basin, and it is difficult to collect a water sample anywhere in the south basin that contains pure phytoplankton without re-suspending sediments. Therefore, we assumed that the isotope signatures of particulate organic matter collected in the pelagic zone of the north basin were representative of those of PPOM in both basins. For RPOM, we also collected water from the river mouths of 29 tributary sites in November 2004. We

found that the inter-annual variation (2004 and 2005) was smaller than the variation across the tributaries (see Appendix of Kohzu et al. 2008). We also used data from November 2004 on the $\delta^{15}N$ values of nitrate in these tributaries (Appendix of Kohzu et al. 2008; Ohte et al. in press).

Lake water samples were filtered through 150-µm nylon mesh and then organic matter was collected on precombusted (450°C for 2 h) glass fiber filters (GF/F, Whattman) as PPOM or RPOM. All food source samples were treated with 0.1 N HCl to remove inorganic carbon and then dried at 60°C for more than 24 h. The foot muscle of snails and the adductor muscle of bivalves were resected and dried at 60°C for more than 24 h. Howard et al. (2005) showed that the $\delta^{15}N$ value of mussels increased by approximately 2‰ with increasing age. In order to examine the relationship between body size and isotope signatures, we compared the dry weight of soft tissues and isotope signatures of macroinvertebrates for sampling sites at which more than three individuals were collected. Dried tissues were ground to a fine powder by mortar and pestle, immersed in a chloroform–methanol mixture (2:1 v/v) for about 24 h to remove lipids, and then re-dried and wrapped in tin capsules for combustion.

Stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) were measured using a continuous-flow isotope ratio mass spectrometer (CF/IRMS) (EA1108, Fisons, Italy; Conflo II and delta S, Finnigan MAT, Germany). Stable isotope ratios are expressed in the δ notation as the differences in parts per thousand (‰) of the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratios of the sample to the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of the standards (Pee Dee Belemnite for $\delta^{13}C$ and atmospheric nitrogen for $\delta^{15}N$). Analytical precision was less than 0.2‰ for both $\delta^{13}C$ and $\delta^{15}N$ in all runs.

Stable isotope mixing model

We used the IsoSource Program (Phillips and Gregg 2003) to estimate the contribution of each food source (PPOM, RPOM, and EOM) to the diet of snails and bivalves using stable isotope data. Trophic enrichment factors were set as +0.8 and +3.4‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively (DeNiro and Epstein 1978; Minagawa and Wada 1984; Vander Zanden and Rasmussen 2001). Our three-source mixing model estimated all possible combinations of each source contribution (0–100%) at increments of 1%. Combinations that fit the observed isotope signatures of macroinvertebrates within the set tolerance ranges were considered feasible solutions (Phillips and Gregg 2003). The initial tolerance was set at 0.5‰; if mixture isotope values were out-of-bounds (i.e., outside of the polygon delineated by the sources; Phillips 2001), the tolerance value was increased by 0.5‰, up to a maximum of 2‰. The output of the model was expressed as the mean and the range (minimum and maximum) of each food source to the diet (e.g., Herrera

et al. 2006; Cardona et al. 2007), where the mean was calculated from all feasible contributions of the source. When analyzing the data, if the difference between maximum and minimum values is small, we can have greater confidence in the results; otherwise, the data should be treated with caution.

Multiple regression analysis

We examined what kinds of human activities in the watershed could have significant effects on the $\delta^{13}C$ and $\delta^{15}N$ signatures of macroinvertebrates using multiple regression analysis. In the present study, we regarded the human population density (HPD) and the fraction of residential area, paddy fields, and other agricultural fields as proxies of human activities. The HPD data were log-transformed, as previously described by Cabana and Rasmussen (1996). The land-use data obtained from the Ministry of Land Infrastructure and Transport (1997), the Ministry of Internal Affairs and Communications (2000), and the Shiga Prefectural Government (2000) were analyzed using Geographic Information System (GIS) software (ArcView ver.8.3, ESRI, CA, USA).

Since there were strong correlations among some geographic parameters (HPD vs. the fraction of residential area, $r = 0.92$; HPD vs. the fraction of paddy fields, $r = 0.63$; the fraction of residential area vs. the fraction of paddy fields, $r = 0.62$), we substituted the residuals of the fraction of residential area, paddy fields, and other agricultural fields against the HPD. After confirming that there was no effect of colinearity on the multiple regression analysis (Graham 1997, 2003), we used the transformed parameters as explanatory variables in the model. The results were obtained using a full model and a reduced model with a forward stepwise method (see Sokal and Rohlf 1995). A set of explanatory variables which could account for most of the site variation in the isotope signatures of snails and bivalves was selected by the stepwise method.

We also examined the influence of water quality in the north and the south basins on the $\delta^{15}N$ value of snails as follows. First, we separated our sampling sites into the south and the north basin groups. Second, we further divided the sampling sites of the north basin group into west and east groups, referring to the classification of the watershed by Nakano et al. (2005) (south basin group: No. 19, 20, 21, 27, 28, 29; north basin-west group: No. 1–6, 25, 26; north basin-east group: No. 7–18, 22, 23, 24 of Fig. 1).

Results

Stable isotope signatures of snails and bivalves

The $\delta^{13}C$ value of snails (Semisulcospira spp.) in the littoral sites near the river mouth (i.e., tributary sites) ranged from −27.6 to −10.6‰, while that for the rock reef sites which are distant from tributaries ranged from −10.7 to −10.6‰ (Table 1). The $\delta^{13}C$ value of bivalves (U. douglasiae biwae) at the tributary sites ranged from −27.5 to −20.3‰, while that for the rock reef sites ranged from −23.7 to −22.1‰. At the rock reef sites, the $\delta^{13}C$ value of snails was similar to that of EOM, and the $\delta^{13}C$ value of bivalves was similar to that of PPOM.

The $\delta^{15}N$ value of snails at the tributary sites varied from 6.6 to 13.9‰, while that for the rock reef sites ranged from 8.2 to 8.9‰ (Table 1). The $\delta^{15}N$ value of bivalves at the tributary sites ranged from 9.1 to 11.9‰, while that for the rock reef sites ranged from 9.9 to 10.6‰. Only the isotope signature of snails at the tributary sites showed large intersite variation.

For the analysis of the relationship between body size and isotope ratios, there was a significant positive correlation for $\delta^{15}N$ value and dry weight of soft tissues at only two of 24 sites for snails and one of 14 sites for bivalves. Similarly, there was a significant negative correlation between $\delta^{13}C$ value and dry weight of soft tissue at only one out of 24 sites for snails. Therefore, in our study, we considered that size-related variations in the isotope ratios of macroinvertebrates contributed little to the variation of isotope ratios at sites.

Stable isotope signatures of potential food sources for macroinvertebrates

The $\delta^{13}C$ and $\delta^{15}N$ values of PPOM were −24.3 and 5.5‰, respectively (Table 1). These values were similar to previous findings (Maki et al. 2010; Yoshimizu et al. unpublished data); those studies showed that isotope ratios of particulate organic matter in the epilimnion (depths of 0, 2.5, 5, and 10 m) at our pelagic zone site (Ie-1; No. 32 of Fig. 1) during September and November 2005 were −25.3 ± 0.9 and 5.1 ± 0.5‰ (mean ± standard deviation), respectively. This similarity of the isotope ratios indicates that the isotope ratios of PPOM were nearly constant at least for this season. Therefore, we used the isotope ratios of PPOM collected in November 2005 for the estimation of the food sources of macroinvertebrates. The stable isotope mixing model estimated the contributions of each food source in the diet of snails and bivalves (Table 2). The results suggested a high contribution of EOM to the snail diet at 17 tributary sites. In contrast, the mixing model showed a low contribution of EOM and relatively high contributions of both PPOM and RPOM to bivalves at 13 tributary sites.

Stable isotopic indicator of human activities

The stepwise multiple regression analysis identified HPD as the significant explanatory variable for the $\delta^{15}N$ value of snails (Table 3). However, for the $\delta^{13}C$ value of snails, no explanatory variable was identified in the stepwise regression model (all $p > 0.1$). We also performed

Table 1 The δ^{13}C and δ^{15}N values of snails (*Semisulcospira* spp.), bivalves (*U. douglasiae biwae*), epilithic organic matter (EOM), pelagic particulate organic matter (PPOM) and riverine particulate organic matter (RPOM) and the δ^{15}N value of nitrate (Kohzu et al. 2008; Ohte et al. in press)

No.	Site	EOM		RPOM		Nitrate	Snails (*Semisulcospira* spp.)			Bivalves (*Unio douglasiae biwae*)		
		δ^{13}C	δ^{15}N	δ^{13}C	δ^{15}N	δ^{15}N	δ^{13}C	δ^{15}N	n	δ^{13}C	δ^{15}N	n
Tributary (Tributary name)												
1	Kamo River.	−10.3	5.1	−27.7	3.5	7.9	−14.2 ± 2.0	9.0 ± 0.2	5	−22.8 ± 0.7	10.1 ± 0.4	6
2	Ado R.	−	−	−23.0	6.2	4.1	−17.1 ± 2.5	6.6 ± 1.3	5	−	−	−
3	Ishida R.	−16.8	8.0	−26.3	4.6	2.9	−	−	−	−	−	−
4	Momose R.	−10.0	5.2	−28.7	6.6	4.6	−17.6 ± 2.4	8.5 ± 1.3	5	−	−	−
5	Oura R.	−10.3	3.7	−28.3	3.0	5.0	−10.6 ± 0.4	8.4 ± 0.3	5	−	−	−
6	Shiotsu-o R.	−13.0	6.3	−28.2	2.2	4.4	−15.2 ± 1.4	8.9 ± 0.3	6	−	−	−
7	Chonoki R.	−18.4	5.9	−26.3	3.5	2.4	−	−	−	−	−	−
8	Ta R.	−15.4	5.5	−26.9	3.6	3.9	−20.0 ± 2.3	8.4 ± 0.3	5	−24	10.1	1
9	Ane R.	−4.9	5.5	−26.6	3.7	3.0	−	−	−	−	−	−
10	Yone R.	−14.7	5.2	−26.1	5.5	5.0	−16.1 ± 0.7	10.0 ± 0.3	5	−23.8 ± 0.5	9.7 ± 0.2	5
11	Amano R.	−12.0	7.6	−25.3	5.5	5.4	−13.1 ± 0.5	9.9 ± 1.9	4	−20.3 ± 0.5	10.6 ± 0.1	5
12	Seri R.	−15.9	7.7	−25.5	6.2	4.3	−	−	−	−	−	−
13	Inukami R.	−11.0	6.7	−25.6	5.2	4.1	−13.9 ± 1.3	9.3 ± 0.3	8	−23.2	11.1	1
14	Ajiki R.	−16.2	7.4	−27.8	4.8	7.0	−	−	−	−	−	−
15	Uso R.	−9.5	8.5	−26.4	4.6	6.0	−17.1 ± 2.8	11.1 ± 0.5	7	−22.9 ± 0.5	10.8 ± 0.4	5
16	Bunroku R.	−	−	−27.1	5.7	8.6	−	−	−	−	−	−
17	Nomazu R.	−	−	−29.0	5.1	7.7	−21.4 ± 2.3	12.8 ± 0.4	3	−26.6 ± 1.2	11.0 ± 0.3	5
18	Echi R.	−12.6	5.2	−27.8	4.0	4.2	−13.2 ± 0.8	9.1 ± 0.2	5	−22.7 ± 0.4	9.1 ± 0.2	3
19	Nagaso R.	−16.8	8.7	−30.5	6.3	10.7	−17.7 ± 2.4	13.1 ± 0.4	5	−	−	−
20	Hayama R.	−15.6	9.0	−28.1	3.9	10.3	−16.7 ± 0.6	12.4 ± 0.2	3	−21.9	7.6	1
21	Shin-moriyama R.	−22.3	10.2	−27.9	4.9	8.4	−21.3 ± 0.6	13.9 ± 0.5	5	−27.1 ± 0.2	11.9 ± 0.6	3
22	Yasu R.	−	−	−28.0	4.4	6.7	−27.6 ± 2.4	7.9 ± 1.9	2	−27.6 ± 1.4	9.5 ± 1.0	3
23	Yanomune R.	−	−	−27.3	4.2	6.6	−22.5 ± 2.5	10.2 ± 1.0	5	−26.2 ± 0.2	10.7 ± 0.3	5
24	Hino R.	−17.4	8.4	−28.5	6.4	9.4	−18.4 ± 1.5	11.4 ± 0.3	5	−24.8 ± 0.4	11.1 ± 0.5	5
25	U R.	−13.1	4.3	−29.3	1.6	2.5	−	−	−	−	−	−
26	Wani R.	−9.1	4.6	−29.0	3.7	5.0	−11.4 ± 1.1	9.4 ± 0.6	5	−22.2 ± 0.2	10.1 ± 0.3	5
27	Mano R.	−8.7	4.6	−29.6	4.0	5.7	−10.8 ± 0.3	9.2 ± 0.8	5	−21.4	10.4	1
28	Tenjin R.	−17.2	4.4	−25.6	4.4	6.0	−13.4 ± 0.9	7.8 ± 0.3	5	−21.1 ± 0.3	10.8 ± 0.2	5
29	Fujinoki R.	−23.5	7.1	−28.2	5.0	5.4	−21.1 ± 3.0	11.3 ± 0.5	5	−22.1	9.3	1
Rock reef												
30	Ohmi-hachiman	−10.1	5.3	−	−	−	−10.6 ± 1.0	8.9 ± 0.5	9	−23.7 ± 2.8	10.6 ± 0.2	3
31	Kaizu-osaki	−10.3	3.8	−	−	−	−10.7 ± 0.2	8.2 ± 0.2	5	−22.1 ± 0.1	9.9 ± 0.1	5
	Pelagic zone			PPOM								
32	Ie-1	−	−	−24.3	5.5	−	−	−	−	−	−	−

The δ^{13}C and δ^{15}N values of snails bivalves are given as mean ± standard deviation. Tributary sites: near the mouths of tributary rivers, rock reef sites: away from tributaries in the littoral zones of Lake Biwa

stepwise multiple regression analysis for the δ^{15}N values of EOM in the littoral zone and nitrate and RPOM in tributaries. Similar to the snail δ^{15}N value, HPD was identified as a significant explanatory variable for the δ^{15}N values of EOM and nitrate, but the squared multiple correlation coefficients (r^2) were lower than that of the model for snails (EOM: $r^2 = 0.25$, nitrate: $r^2 = 0.39$, snails: $r^2 = 0.54$; Fig. 2). The δ^{15}N value of RPOM was not significantly explained by HPD or any land-use pattern ($p > 0.1$). We also examined the relationships among the δ^{15}N values of snails, EOM, and nitrate, and found that there were significant positive correlations between the δ^{15}N values of nitrate and EOM ($r = 0.55$, $p = 0.005$) and between the δ^{15}N values of EOM and snails ($r = 0.90$, $p < 0.0001$).

The HPD significantly differed among geographical groups of the watershed (i.e., south basin, north basin-west, and north basin-east groups; one-way ANOVA: $F_{2,26} = 26.58$, $p < 0.001$, Tukey's post hoc test: all

$p < 0.005$). However, there were no significant differences among groups in the residuals of the δ^{15}N values of snails, EOM, and nitrate against HPD (one-way ANOVA, snails: $F_{2,19} = 0.09$, $p > 0.1$, EOM: $F_{2,21} = 0.29$, $p > 0.1$, nitrate: $F_{2,26} = 0.42$, $p > 0.1$). This result indicates that there were no differences in the δ^{15}N values among geographical areas but that the values were dependent on the inputs from each watershed.

The δ^{15}N value of bivalves was not significantly explained by HPD or any of the land-use patterns ($p > 0.1$), although the δ^{13}C value of bivalves was marginally related to the fraction of paddy fields (Table 3). We also performed stepwise multiple regression analysis for the δ^{13}C value of RPOM in tributaries. However, the δ^{13}C value of RPOM was not significantly explained by HPD or any of the land-use patterns ($p > 0.1$; Fig. 3). There was no significant correlation between the δ^{13}C values of bivalves and RPOM ($p > 0.1$).

Table 2 Percent contributions (mean, with minimum–maximum ranges) of food sources to diet of (a) snails (*Semisulcospira* spp.) and (b) bivalves (*U. douglasiae biwae*), calculated using the IsoSource Program

No.	Tributary name	Snails (*Semisulcospira* spp.)					
		PPOM (%)		RPOM (%)		EOM (%)	
(a)							
1	Kamo River	30	(24–37)	3	(0–7)	67	(63–71)
4	Momose R.	45	(26–62)	11	(0–23)	45	(38–51)
5	Oura R.	6	(0–18)	5	(0–14)	89	(82–100)
6	Shiotsu-o R.	11	(0–28)	12	(2–23)	77	(70–83)
8	Ta R.	31	(0–66)	24	(0–51)	46	(34–57)
10	Yone R.	13	(0–38)	11	(0–32)	76	(62–93)
11	Amano R.	8	(0–23)	8	(0–21)	84	(77–93)
13	Inukami R.	14	(0–31)	13	(0–28)	73	(69–78)
15	Uso R.	33	(0–60)	18	(0–46)	49	(40–56)
18	Echi R.	11	(7–16)	1	(0–3)	88	(84–92)
19	Nagaso R.	5	(0–15)	7	(0–20)	88	(80–98)
20	Hayama R.	11	(0–28)	7	(0–19)	82	(72–92
21	Shin-moriyama R.	2	(0–4)	1	(0–3)	98	(96–100)
24	Hino R.	13	(0–31)	8	(0–20)	78	(69–88)
26	Wani R.	13	(0–30)	7	(0–17)	81	(70–91)
27	Mano R.	24	(23–25)	0	(0–1)	76	(75–77)
29	Fujinoki R.	17	(0–50)	3	(0–8)	80	(50–100)

No.	Tributary name	Bivalves (*Unio douglasiae biwae*)					
		PPOM (%)		RPOM (%)		EOM (%)	
(b)							
1	Kamo River	85	(71–100)	7	(0–15)	8	(0–18)
10	Yone R.	42	(0–100)	51	(0–95)	8	(0–21)
11	Amano R.	24	(0–66)	41	(0–66)	35	(34–39)
13	Inukami R.	45	(0–83)	28	(0–66)	28	(17–37)
15	Uso R.	85	(84–86)	1	(0–2)	14	(14–14)
18	Echi R.	82	(68–97)	9	(0–18)	9	(3–15)
20	Hayama R.	46	(0–98)	35	(0–68)	19	(2–35)
21	Shin-moriyama R.	3	(0–8)	65	(62–69)	32	(30–35)
24	Hino R.	8	(0–24)	67	(50–82)	26	(17–35)
26	Wani R.	86	(74–100)	5	(0–14)	9	(0–20)
27	Mano R.	74	(55–99)	11	(0–27)	16	(1–31)
28	Tenjin R.	92	(91–94)	1	(0–2)	7	(6–9)
29	Fujinoki R.	23	(2–50)	3	(0–8)	75	(50–90)

We initially used a small initial tolerance (0.5‰) and then increased it by 0.5 up to 2‰
PPOM pelagic particulate organic matter, *RPOM* riverine particulate organic matter, *EOM* epilithic organic matter

Table 3 The full model and the reduced model using stepwise multiple regression analysis: (a) the δ^{15}N value of snails (*Semisulcospira* spp.), (b) the δ^{13}C value of bivalves (*U. douglasiae biwae*)

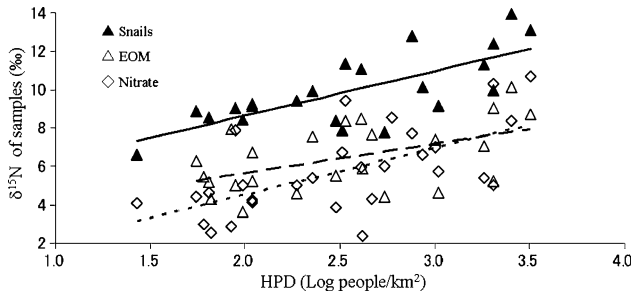| Explanatory variable	Full			Reduced		
	Coefficient	Standard error	p value	Coefficient	Standard error	p value
(a)						
Intercept	4.01	1.27	0.01	4.01	1.25	0.004
Human population density (HPD)	2.32	0.49	<0.001	2.32	0.48	<0.001
Residual of the fraction of paddy fields	0.87	2.02	0.67			
Residual of the fraction of other agricultural fields	−20.63	35.84	0.57			
Residual of the fraction of residential area	7.57	6.80	0.28			
	$r^2 = 0.60$, $F_{1,20} = 6.33$, $p = 0.003$			$r^2 = 0.54$, $F_{1,20} = 23.35$, $p < 0.001$		
(b)						
Intercept	−21.11	2.94	<0.001	−23.57	0.49	<0.001
Human population density (HPD)	−0.92	1.08	0.41			
Residual of the fraction of paddy fields	−5.95	3.79	0.14	−6.93	3.44	0.06
Residual of the fraction of other agricultural fields	−81.07	64.66	0.23			
Residual of the fraction of residential area	−7.89	13.93	0.58			
	$r^2 = 0.34$, $F_{1,15} = 1.56$, $p = 0.25$			$r^2 = 0.21$, $F_{1,15} = 4.06$, $p = 0.06$		

Fig. 2 Relationships between human population density (HPD; people/km^2) in the watershed of the tributary rivers and the δ^{15}N values of snails (*Semisulcospira* spp.) (*closed triangles*), epilithic organic matter (EOM) (*open triangles*) in littoral sites near the mouths of the tributaries, and nitrate (*open diamonds*; Kohzu et al. 2008; Ohte et al. in press) in the tributaries. HPD was transformed into logarithmic values. Regression lines are indicated if significant; δ^{15}N value of snails (*solid line*): δ^{15}N = 4.01 + 2.32 × log (HPD) (r^2 = 0.54, p < 0.001), δ^{15}N value of EOM (*dashed line*): δ^{15}N = 2.62 + 1.52 × log (HPD) (r^2 = 0.25, p = 0.01) and δ^{15}N value of nitrate (*dotted line*): δ^{15}N = −0.29 + 2.42 × log (HPD) (r^2 = 0.39, p < 0.001)
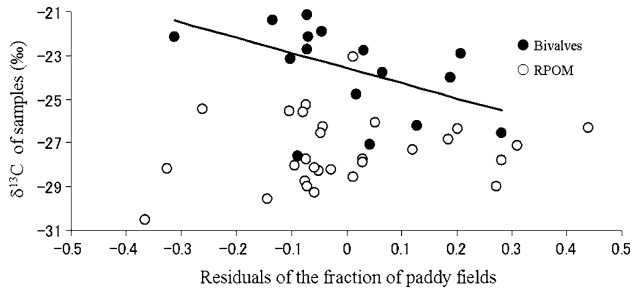

Fig. 3 Relationship between fraction of paddy fields in the watershed of the tributaries and the δ^{13}C values of bivalves (*U. douglasiae biwae*) (*closed circles*) in littoral sites near the mouths of the tributaries and riverine particulate organic matter (*RPOM*) (*open circles*) in the tributaries. A marginally significant regression line was found for the δ^{13}C value of bivalves (*solid line*): δ^{13}C = −23.57 − 6.93 × fraction of paddy fields (r^2 = 0.21, p = 0.06)

Discussion

The isotope mixing model indicates that the major food source of snails (*Semisulcospira* spp.) was EOM at the tributary sites. In addition, at the rock reef sites, the δ^{13}C value of snails was also similar to that of EOM, suggesting that they fed exclusively on EOM. The δ^{15}N values of snails, EOM, and nitrate were positively correlated with HPD (Table 3; Fig. 2), as reported in previous studies on the tributaries of Lake Biwa (Kohzu et al. 2008; Kohzu et al. 2009) and other lakes (Cabana and Rasmussen 1996). Moreover, there were significant positive correlations between the δ^{15}N values of snails, EOM, and nitrate. These results suggest that EOM in the littoral zone assimilates nitrogen derived from human activities in the watershed that is then accumulated in snails through consumption of EOM.

The squared multiple correlation coefficient between HPD and the δ^{15}N value of snails was higher than those between HPD and the isotope ratios of nitrate and EOM (Fig. 2). Because the isotopic turnover rate of macroinvertebrates is slower than that of primary producers (Cabana and Rasmussen 1996), the δ^{15}N value of snails can be used as an effective and reliable indicator of nitrogen loading from the watershed. The δ^{15}N value of macroinvertebrates has already been shown to be a useful indicator of nitrogen loading from the watershed in various lakes and ponds (Cabana and Rasmussen 1996; Lake et al. 2001; McKinney et al. 2002; Vander Zanden et al. 2005). However, the present study has uniquely shown that, even within a lake, the base of the local food web in the littoral zone is influenced by anthropogenic loading through the tributary rivers. Cabana and Rasmussen (1996) suggested that denitrification also influences the δ^{15}N value of organisms in aquatic systems. In Lake Biwa, Yamada et al. (1996) showed that δ^{15}N value of nitrate in the pelagic zone of the north basin was higher than that of a tributary river (Ane River; No. 9 of Fig. 1) due to denitrification in the north basin. In this study, we did not examine denitrification activity in each littoral zone, and therefore we cannot discuss the influence of denitrification on the variation in δ^{15}N value of the organisms. In contrast to δ^{15}N, δ^{13}C value of snails was not explained by HPD or any land-use pattern probably because the diet of snails was mainly EOM, and RPOM, which may contain anthropogenic organic matter from the watershed, makes little contribution to the diet of snails.

The isotope mixing model indicates that the food sources of bivalves include both PPOM and RPOM in the tributary sites (Table 2), while the diet is mainly PPOM in the rock reef sites (Table 1). The δ^{15}N values of bivalves and RPOM were not correlated with HPD. In addition, the variation in the δ^{15}N value of bivalves (range 9.1–11.9‰) was smaller than that of snails (6.6–13.9‰). These results suggest that the δ^{15}N value of bivalves was less influenced by anthropogenic loading than that of snails, which assimilate inorganic nitrogen from the watershed through a diet composed mainly of EOM.

The δ^{13}C value of bivalves was marginally related to the fraction of paddy fields (r^2 = 0.21) (Table 3; Fig. 3), suggesting that the δ^{13}C value of bivalves may be related to agricultural drainage from paddy fields. The δ^{13}C value of RPOM, one of the food sources of bivalves, was not correlated with HPD or any land-use patterns (Fig. 3). Moreover, there was no significant relationship between the δ^{13}C values of bivalves and RPOM. The amount of RPOM changes seasonally, with RPOM typically containing organic matter derived from agricultural drainage in May, as a result of the intensive flooding of paddy fields (Ohkubo and Azuma. 2005). The isotope ratios of bivalves collected in November (non-flooding period) might not clearly reflect the temporal influence of agricultural drainage from paddy fields in May, because bivalves integrate the seasonal changes in food sources over much longer time periods.

In conclusion, our findings show that $\delta^{13}C$ and $\delta^{15}N$ signatures of macroinvertebrates inhabiting the littoral zone near the mouths of tributary rivers are useful indicators of anthropogenic impacts from the watershed. In particular, $\delta^{15}N$ value of snails is a useful indicator of inorganic nitrogen loading from the watershed. Our results also show that one of the important food sources of bivalves is RPOM and that the $\delta^{13}C$ value of bivalves is an indicator of temporal organic carbon loading from the watershed (e.g., paddy fields). However, we need to consider the turnover rates of the tissue of bivalves and sampling period in order to detect the influence of temporal organic carbon loadings from the watershed on the isotope signatures of bivalves. The amount of loading from the watershed to the lake fluctuates over time, especially during the flooding period and the rainy and typhoon seasons (Ebise 2000; Ohkubo and Azuma 2005). Therefore, frequent monitoring of nutrient concentrations in tributaries and the lake water is required. As long-lived macroinvertebrates integrate $\delta^{13}C$ and $\delta^{15}N$ signatures of primary producers over long periods of time (Cabana and Rasmussen 1996, Post 2002), their isotope signatures are useful indicators of anthropogenic impacts from the watershed, not only for comparison among lakes (e.g., Cabana and Rasmussen 1996; Lake et al. 2001; McKinney et al. 2002; Vander Zanden et al. 2005) but also for comparisons among heterogeneous environments in the littoral zone within a lake.

Appendix

See Table 4.

Table 4 Human population density (HPD; people/km^2), fraction of paddy fields, fraction of other agricultural fields and fraction of residential area in the watersheds of individual tributary rivers to Lake Biwa

No.	Tributary name	HPD (log people/km^2)	Fraction of paddy fields	Fraction of other agricultural fields	Fraction of residential area
1	Kamo River	1.95	0.18	0.00	0.01
2	Ado R.	1.43	0.03	0.01	0.01
3	Ishida R.	1.92	0.10	0.01	0.02
4	Momose R.	1.81	0.04	0.00	0.01
5	Oura R.	1.99	0.11	0.02	0.03
6	Shiotsu-o R.	1.74	0.07	0.00	0.02
7	Chonoki R.	2.62	0.76	0.04	0.14
8	Ta R.	2.48	0.47	0.02	0.10
9	Ane R.	1.78	0.06	0.01	0.02
10	Yone R.	3.31	0.55	0.01	0.35
11	Amano R.	2.35	0.18	0.02	0.06
12	Seri R.	2.66	0.07	0.01	0.08
13	Inukami R.	2.04	0.07	0.01	0.03
14	Ajiki R.	3.00	0.70	0.01	0.20
15	Uso R.	2.61	0.52	0.01	0.11
16	Bunroku R.	2.77	0.67	0.01	0.12
17	Nomazu R.	2.88	0.66	0.01	0.20
18	Echi R.	2.04	0.10	0.01	0.03
19	Nagaso R.	3.50	0.18	0.02	0.31
20	Hayama R.	3.31	0.44	0.01	0.30
21	Shin-moriyama R.	3.40	0.55	0.00	0.34
22	Yasu R.	2.51	0.20	0.03	0.07
23	Yanomune R.	2.93	0.52	0.01	0.16
24	Hino R.	2.53	0.31	0.02	0.08
25	U R.	1.82	0.06	0.00	0.01
26	Wani R.	2.27	0.16	0.00	0.05
27	Mano R.	3.02	0.28	0.00	0.16
28	Tenjin R.	2.73	0.27	0.01	0.07
29	Fujinoki R.	3.26	0.16	0.01	0.21

References

Cabana G, Rasmussen JB (1996) Comparison of aquatic food chains using nitrogen isotopes. Proc Natl Acad Sci USA 93:10844–10847

Cardona L, Revelles M, Sales M, Aguilar A, Borrell A (2007) Meadows of the seagrass *Posidonia oceanica* are a significant source of organic matter for adjoining ecosystems. Mar Ecol Prog Ser 335:123–131

Deniro MJ, Epstein S (1978) Influence of diet on the distribution of carbon isotopes in animals. Geochim Cosmochim Acta 42:495–506

Ebise S (2000) Inflowing river. In: Somiya I (ed) Lake Biwa: environment and water quality formation (in Japanese). Gihodo Shuppan, Tokyo, pp 53–65

France RL (1995a) Carbon-13 enrichment in benthic compared to planktonic algae: foodweb implications. Mar Ecol Prog Ser 124:307–312

France RL (1995b) Differentiation between littoral and pelagic food webs in lakes using stable carbon isotopes. Limnol Oceanogr 40:1310–1313

France RL (1996) Scope for use of stable carbon isotopes in discerning the incorporation of forest detritus into aquatic foodwebs. Hydrobiologia 325:219–222

Graham MH (1997) Factors determining the upper limit of giant kelp, *Macrocystis pyrifera* Agardh, along the Monterey Peninsula, central California, USA. J Exp Mar Biol Ecol 218:127–149

Graham MH (2003) Confronting multicollinearity in ecological multiple regression. Ecology 84:2809–2815

Herrera MLG, Hobson KA, Martínez JC, Méndez CG (2006) Tracing the origin of dietary protein in tropical dry forest birds. Biotropica 38:735–742

Howard JK, Cuffey KM, Solomon M (2005) Toward using *Margaritifera falcata* as an indicator of base level nitrogen and carbon isotope ratios: insights from two California Coast Range rivers. Hydrobiologia 541:229–236

Kohzu A, Miyajima T, Tayasu I, Yoshimizu C, Hyodo F, Matsui K, Nakano T, Wada E, Fujita N, Nagata T (2008) Use of stable nitrogen isotope signatures of riparian macrophytes as an indicator of anthropogenic N inputs to river ecosystems. Environ Sci Technol 42:7837–7841

Kohzu A, Tayasu I, Yoshimizu C, Maruyama A, Kohmatsu Y, Hyodo F, Onoda Y, Igeta A, Matsui K, Nakano T, Wada E, Nagata T, Takemon Y (2009) Nitrogen-stable isotopic signatures of basal food items, primary consumers and omnivores in rivers with different levels of human impact. Ecol Res 24:127–136

Lake JL, McKinney RA, Osterman FA, Pruell RJ, Kiddon J, Ryba SA, Libby AD (2001) Stable nitrogen isotopes as indicators of anthropogenic activities in small freshwater systems. Can J Fish Aquat Sci 58:870–878

Maki K, Kim C, Yoshimizu C, Tayasu I, Miyajima T, Nagata T (2010) Autochthonous origin of semi-labile dissolved organic carbon in a large monomictic lake (Lake Biwa): Carbon stable isotopic evidence. Limnology. doi:10.1007/s10201-009-0299-z

McClelland JW, Valiela I (1998) Linking nitrogen in estuarine producers to land-derived sources. Limnol Oceanogr 43:577–585

McClelland JW, Valiela I, Michener RH (1997) Nitrogen-stable isotope signatures in estuarine food webs: a record of increasing urbanization in coastal watersheds. Limnol Oceanogr 42:930–937

McKinney RA, Lake JL, Allen M, Ryba S (1999) Spatial variability in Mussels used to assess base level nitrogen isotope ratio in freshwater ecosystems. Hydrobiologia 412:17–24

McKinney RA, Lake JL, Charpentier MA, Ryba S (2002) Using mussel isotope ratios to assess anthropogenic nitrogen inputs to freshwater ecosystems. Environ Monit Assess 74:167–192

Minagawa M, Wada E (1984) Stepwise enrichment of ^{15}N along food chains: Further evidence and the relation between $\delta^{15}N$ and animal age. Geochim Cosmochim Acta 48:1135–1140

Ministry of Internal Affairs and Communications (2000) Population Census, Statistics Bureau, Tokyo

Ministry of Land Infrastructure and Transport (1997) Digital National Land Information, Tokyo

Mori S, Yamamoto K, Negoro K, Horie S, Suzuki N (1967) First report of the regular limnological survey of Lake Biwa (Oct. 1965–Dec. 1966) I. General remark. Mem Fac Sci Kyoto Univ Ser Biol 1:36–40

Nakanishi M, Sekino T (1996) Recent drastic changes in Lake Biwa bio-communities, with special attention to exploitation of the littoral zone. GeoJournal 40:63–67

Nakano T, Tayasu I, Wada E, Igeta A, Hyodo F, Miura Y (2005) Sulfur and strontium isotope geochemistry of tributary rivers of Lake Biwa: implications for human impact on the decadal change of lake water quality. Sci Total Environ 345:1–12

Ohkubo T, Azuma Y (2005) Load of turbid from the watershed of Lake Biwa and its effect on the lake water quality. In: Memorial volume of the Lake Biwa Research Institute (in Japanese). Lake Biwa Research Institute, Otsu, Shiga, pp 55–72

Ohte N, Tayasu I, Kohzu A, Yoshimizu C, Osak K, Makabe A, Koba K, Yoshida N, Nagata T (in press) Spatial distribution of nitrate sources of rivers in the Lake Biwa watershed, Japan: controlling factors revealed by nitrogen and oxygen isotope values. Water Resour Res. doi:10.1029/2009WR007871

Okuda S, Kumagai M (1995) Introduction. In: Okuda S, Imberger J, Kumagai M (eds) Physical processes in a large lake: Lake Biwa, Japan. Am Geophys Union, Washington, DC, pp 1–6

Peterson BJ, Fry B (1987) Stable isotopes in ecosystem studies. Ann Rev Ecol Syst 18:293–320

Phillips DL (2001) Mixing models in analyses of diet using multiple stable isotopes: a critique. Oecologia 127:166–170

Phillips DL, Gregg JW (2003) Source partitioning using stable isotopes: coping with too many sources. Oecologia 136:261–269

Post DM (2002) Using stable isotopes to estimate trophic position: models, methods, and assumptions. Ecology 83:703–718

Shiga Prefectural Government (2000) Watershed map of Shiga Prefecture. Otsu, Shiga

Shiga Prefectural Government (2008) White paper on the environment of Shiga Prefecture (in Japanese). Shiga Prefectural Government, Otsu, Shiga

Shiga Prefectural Government (2009) River and harbors in Shiga Prefecture (in Japanese). Otsu, Shiga

Sokal RR, Rohlf FJ (1995) Choosing predictor variables. In: Biometry: the principles and practice of statistics in biological research, 3rd edn. Freeman, New York, pp 654–664

Thorp JH, Covich AP (1991) Ecology and classification of North American freshwater invertebrates. Academic Press, San Diego

Vander Zanden MJ, Rasmussen JB (1999) Primary consumer $\delta^{13}C$ and $\delta^{15}N$ and the trophic position of aquatic consumers. Ecology 80:1395–1404

Vander Zanden MJ, Rasmussen JB (2001) Variation in $\delta^{15}N$ and $\delta^{13}C$ trophic fractionation: implications for aquatic food web studies. Limnol Oceanogr 46:2061–2066

Vander Zanden MJ, Vadeboncoeur Y, Diebel MW, Jeppesen E (2005) Primary consumer stable nitrogen isotopes as indicators of nutrient source. Environ Sci Technol 39:7509–7515

Yamada Y, Ueda T, Wada E (1996) Distribution of carbon and nitrogen isotope ratios in the Yodo River watershed. Jpn J Limnol 57:467–477

Yoshii K (1999) Stable isotope analyses of benthic organisms in Lake Baikal. Hydrobiologia 411:145–159