Hypoxia within macrophyte vegetation limits the use of methane-derived carbon by larval chironomids in a shallow temperate eutrophic lake

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Abstract

Methane-derived carbon (MDC) can subsidize lake food webs. However, the trophic transfer of MDC to consumers within macrophyte vegetation is largely unknown. We investigated the seasonality of $\delta^{13}C$ in larval chironomids within *Nelumbo nucifera* (Gaertn.) and *Trapa natans* var. *Japonica* (Nakai) vegetation in the shallow, eutrophic Lake Izunuma in Japan. Over the last several years, *N. nucifera* has rapidly expanded across more than 80% of the lake surface. Prior to the expansion of *N. nucifera* (2007–2008), a previous study reported extremely low larval $\delta^{13}C$ levels with peak sediment methane concentrations in August or September. After the expansion of *N. nucifera* (2014–2015), we observed extreme hypoxia as low as or lower than 1 mg l^{-1} among the macrophyte coverage during June and August. During August and September, no larvae could be found among *N. nucifera* and larvae in *T. natans* showed relatively high $\delta^{13}C$ levels (> −40‰). In contrast, larvae were markedly ^{13}C–depleted (down to −60‰) during October and November. The renewed supply of oxygen to the lake bottom may stimulate MOB activity, leading to an increase in larval assimilation of MDC. Our results suggest that macrophyte vegetation can affect the seasonality of MDC transfer to benthic consumers under hypoxic conditions in summer.

Introduction

Recent studies have provided evidence that methane-derived carbon (MDC) can subsidize food webs in lake ecosystems (Kiyashko et al., 2001; Grey et al., 2004a; Jones et al., 2008; Ravinet et al., 2010; Jones & Grey, 2011). Due to isotopic fractionation during methanogenesis, biogenic methane is typically extremely ^{13}C-depleted (−80 to −60‰; Whiticar, 1999) compared with other food sources available to aquatic consumers: allochthonous organic matter (−28 to −26‰; Peterson & Fry, 1987), and autochthnous organic matter (typically ranging from −35 to −25‰; Post, 2002). Isotopic fractionation during the biological oxidation of methane by methane-oxidizing bacteria (MOB) can lead to further isotopic depletion of microbial carbon (Whiticar, 1999). Thus, markedly low $\delta^{13}C$ levels in benthic invertebrates (mainly larval chironomids) reflect the assimilation of MDC by these organisms through the consumption of MOB (for a review, see Jones & Grey, 2011, Grey, 2016). The use of MDC by larval chironomids has often been reported in stratified lakes in which oxygen can be depleted near the lake bottom. In contrast, MDC tends to be less assimilated by larvae in shallow lakes where the entire water column is mixed frequently, keeping oxygen in contact with the sediments (Grey et al., 2004b; Jones et al., 2008). However, several studies have indicated that biogenic methane can be an important carbon source for consumers in shallow lakes (Sanseverino et al., 2012; Yasuno et al., 2012; Agasild et al., 2014).

Shallow mesotrophic and eutrophic lakes can often present contrasting states: a clear-water state that is dominated by submersed macrophytes, and a turbid-water state that is dominated by phytoplankton (Scheffer et al., 1993; Moss et al., 1994; Hargeby et al., 2007; Scheffer & Jeppesen, 2007). The former is considered to be the pristine state

for the majority of shallow lakes, because macrophytes can support a diversity of lacustrine organisms by providing food and habitat (Carpenter & Lodge, 1986; Jeppesen et al., 1998; Scheffer, 1998). Macrophyte vegetation can maintain a clear-water state via various mechanisms including stabilizing sediments, releasing allelopathic substances, and promoting zooplankton populations by providing refuge (Scheffer et al., 1993; Jeppesen et al., 1998; Scheffer, 1998; Hargeby et al., 2004; Hilt & Gross, 2008). As nutrient loads increase, the dominant primary producers can shift from submersed macrophytes to taller submersed species and floating-leaved rooted plants (Wetzel, 2001b). Further nutrient loading can result in a regime shift to a state dominated by phytoplankton, although threshold nutrient levels that induce this shift depend on lake size, depth and climate (Scheffer & van Nes, 2007).

Aquatic macrophytes can strongly affect dissolved oxygen (DO) concentrations in the water column in shallow waters (Rose & Crumpton, 2006; Yamaki & Yamamuro, 2013). Macrophyte vegetation supplies a large amount of detritus to the sediment (Carpenter, 1981), and aquatic macrophytes typically reduce water circulation and sediment resuspension (Dieter, 1990). Decomposition of detritus in the sediment can increase oxygen demand, especially during summer when water temperature increases, thereby depleting DO (Webster & Benfield, 1986). Floating-leaved and emergent plants can prevent gas exchange between the water surface and the air and inhibit primary production by phytoplankton (Frodge et al., 1990; Caraco et al., 2006), resulting in strong oxygen depletion (<1 mg l^{-1}; Turner et al., 2010; Yamaki & Yamamuro, 2013).

Low-oxygen conditions in lakes can enhance methane cycles (*i.e.*, methane production and oxidation), resulting in a greater biomass of MOB available for consumption by larval chironomids (Deines et al., 2007b; Gentzel et al., 2012; Hershey

et al., 2015). In fact, ^{13}C-depleted larval chironomids have often been reported in lakes in which the oxygen concentration near the lake bottom dropped below 2 mg l^{-1} in late summer (Jones et al., 2008). Therefore, dense macrophyte vegetation may enhance the trophic transfer of MDC to benthic consumers. In contrast, larval chironomids aestivate under low oxygen conditions (< 1 mg l^{-1}; Hamburger et al., 1994). Anoxia near the lake bottom can restrict microbial methane oxidation and may prevent MOB from multiplying, resulting in relatively small amounts of biomass available to benthic consumers (Jones & Grey, 2011; Child & Moore, 2015). Thus, the effects of aquatic vegetation on the contribution of MDC to benthic consumers appear to be controversial. Complementary studies on the trophic transfer of MDC under the influence of aquatic vegetation will provide a better understanding of food web dynamics and the carbon cycle in wetlands.

Lake Izunuma is a temperate, eutrophic, and shallow lake in Japan. Approximately 40% of the lake surface was covered by lotus (*Nelumbo nucifera*) in 2007. Since then, the lotus coverage has expanded to cover more than 80% of the water surface (Shikano S., unpublished data, Fig. 1). In addition, floating-leaved plants such as *Trapa* spp. dominate outside of the lotus vegetation, and open areas are rare. Macrophyte coverage caused extreme depletion of DO during summer (Yasuno et al., 2015). Before the expansion of the lotus vegetation, the contribution of MDC to larval *Chironomus plumosus* L. (Diptera: Chironomidae) peaked simultaneously with the methane concentration in the sediment in August or September. During this time, frequent water circulation supplied oxygen to the sediment surface and DO concentrations above the lake bottom were greater than 2 mg l^{-1} (Yasuno et al., 2012). However, hypoxia associated with macrophyte vegetation can affect the MDC pathway

114 to benthic consumers positively and/or negatively. Hypoxia may promote microbial
115 methane oxidation and increase the biomass of MOB available to larval chironomids
116 (Hershey et al., 2015). In addition, the accumulation of organic matter derived from
117 macrophytes on the sediment may also promote methane cycles (Chan et al., 2005;
118 Schwarz et al., 2008). In contrast, extreme hypoxia (< 1 mg l^{-1}) or anoxia can render
119 larvae inactive or make the lake bottom too harsh an environment for their survival.
120 Lake Izunuma is thus an ideal site at which to investigate the effects of macrophyte
121 vegetation on the trophic transfer of MDC by comparing isotopic data obtained from
122 larval chironomids before and after the expansion of *N. nucifera* vegetation.
123 The purpose of this study was to test the following hypotheses: (1) hypoxia
124 associated with macrophyte vegetation limits the use of MDC by benthic consumers
125 during late summer (August) and early autumn (September), and (2) the use of MDC
126 increases in autumn (October or November) when DO is supplied to the sediment-water
127 interface. The results of the current study are compared with those of a previous study
128 (Yasuno et al., 2012) in order to assess the effects of macrophyte vegetation on the
129 trophic transfer of MDC to benthic invertebrates.
130
131 **Materials and Methods**
132 *Study site*
133 Lake Izunuma is located in northeastern Honshu, Japan (38°43' N, 141°06' E; Fig. 1). It
134 is a temperate, eutrophic, shallow lake (maximum depth of approximately 1.6 m, area of
135 3.69 km^2) situated 6 m above sea level (Shidara, 1992). During summer and early
136 autumn (June and September), a significant part of the water surface is usually covered
137 by the lotus *N. nucifera*, which is a floating-leaved emergent macrophyte. Other

138 floating-leaved macrophytes, such as *Trapa japonica* Flerow, *Trapa Natans* var.
139 *japonica* Nakai, *Nymphoides indica* (L.) O. Kuntze, and *Nymphoides peltat*a (S.G.
140 Gmel.) Kuntze have also been identified on the lake surface (The Miyagi Prefectual
141 Izunuma-Uchinuma Environmental Foundation, 2010). The lotus typically undergoes a
142 population cycle in which it is nearly eliminated by the submergence of its floating
143 leaves in flood, followed by a population expansion lasting 15–20 years
144 (Izunuma-Uchinuma Natural regeneration council, 2009). The last flood occurred in the
145 summer of 1998. Since then, water levels have not significantly risen and the lotus
146 population has been continuously expanding. The lotus covered approximately 40% of
147 the water surface in 2007 and 2008. During recent years, the lotus has expanded to
148 cover more than 80% of the water surface and most of the water surface outside of the
149 lotus-covered area has been colonized by other floating-leaved plants (Shikano
150 unpublished data). Lotus on Lake Izunuma begins to wither in October and the withered
151 petioles without leaves often remain until the following spring. The water surface may
152 be covered by ice during winter. *C. plumosus* dominates the benthic fauna in the
153 profundal zone (Yasuno et al., 2009; Yasuno et al., 2015). Prior to the expansion of lotus,
154 annual averages of total nitrogen were 0.74 mg l^{-1} in 2007 and 0.89 mg l^{-1} in 2008, and
155 those of total phosphate were 0.08 mg l^{-1} in 2007 and 0.10 mg l^{-1} in 2008 (National
156 Institute of Environmental Studies, 2017). After the expansion of lotus, annual averages
157 of total nitrogen (0.72 mg l^{-1} in 2014 and 0.68 mg l^{-1} in 2015) and total phosphate (0.06
158 mg l^{-1} in 2014 and 0.08 mg l^{-1} in 2015) slightly decreased (Miyagi Prefecture, 2017),
159 but remained within the range of eutrophic lakes (Wetzel, 2001a).
160
161 *Field survey*

We conducted surveys at two sites, designated Site A (within an area covered by lotus *N. nucifera* vegetation) and Site B (within an area dominated by *T. natans* vegetation), monthly from June to December 2014, and from March to September 2015 (Fig. 1), to compare the effects of lotus and *T. natans* vegetation on the use of MDC by larval chironomids as well as DO concentrations and methane concentrations in the sediment. Yasuno et al. (2012) surveyed at the same site as Site B, but in the absence of macrophytes, from June 2007 to September 2008. Thus, we designated the site surveyed by Yasuno et al. (2012) as Site C to evaluate the effects of *T. natans* vegetation on the use of MDC by chironomid larvae. Temperature and DO concentrations were determined at the lake surface and near the lake bottom (10–30 cm above lake bottom) using an HQ30d Portable Optical Dissolved Oxygen Meter (Central Kagaku Corp., Tokyo, Japan). We collected samples of larval chironomids and their potential food sources (particulate organic matter (POM) and sediment) for stable isotope analyses and core samples of sediments to measure methane concentrations. In September 2014 and April 2015, we collected only core samples for methane. In August 2015, we collected samples only for stable isotope analyses.

Sampling of larval chironomids and their potential food sources

Fourth-instar larvae of *C. plumosus* were collected using an Ekman grab sampler and sieved from the surrounding sediment (mesh size: 1 mm). We used fourth instar larvae of *C. plumosus* in order to compare our data with those of previous studies that measured $\delta^{13}C$ levels in fourth instar *C. plumosus* (Grey et al., 2004b; Deines et al., 2007b). Ekman grab sampling was repeated at least 20 times per site during each survey. In total, 5–16 larval individuals were collected, except in August and/or September

(Table 1). No larvae were collected from Site A in August 2014, August 2015, or September 2015. We did not measure stable isotope ratios of larval chironomids from Site B in September 2015, since we could obtain only one individual. Larvae were transported to the laboratory and maintained alive in filtered lake water for at least 24 h in order to eliminate their gut contents. Fecal matter was removed periodically to prevent coprophagy (Grey et al., 2004b). Larvae were freeze-dried (24 h), ground and homogenized using an agate mortar and pestle, and treated with a chloroform–methanol mixture (2:1 by volume) to remove lipids (Yoshii et al., 1999), which are depleted in ^{13}C compared to proteins and carbohydrates (Deniro & Epstein, 1977). The samples were then concentrated onto GF/C glass filters (precombusted at 500°C for 2 h; Whatman, Florham Park, NJ, USA) and freeze-dried. Surface sediment was collected using an Ekman grab sampler. We collected three replicates of surface lake water for POM samples. The samples were preserved in crushed ice and transported to the laboratory. Sediment samples were dried (60°C, 24 h) and treated with 1 N HCl, washed with distilled water, dried in a 60°C oven (24 h), ground, homogenized, and subjected to stable isotope analyses.

Stable isotope analyses

Stable isotope ratios were determined with a mass spectrometer (Delta V Advantage; Thermo Electron Corp., San Diego, CA, USA) connected to an elemental analyzer (Flash 2000; CE Instruments Ltd., Wigan, UK). Stable isotope ratios are represented using the standard delta notation,

$$\delta^{13}C \text{ or } \delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1{,}000\ (‰),$$

where R = $^{13}C/^{12}C$ or $^{15}N/^{14}N$. We report isotopic values relative to the following standards: Pee Dee belemnite for $\delta^{13}C$ and nitrogen gas for $\delta^{15}N$. The analytical error was within ±0.1‰ for carbon and ±0.2‰ for nitrogen.

Sediment methane concentrations

To measure methane concentrations in sediments, three sediment cores were collected using a long pipe equipped with a PVC column (5-cm diameter). Approximately 5 mL of sediment subsample were collected from each section of sediment core, 0–1 cm and 5–6 cm, at both sites. The two subsamples from each of three different cores were put into 50-mL gastight vials (SVG-50; Nichiden-Rika Glass Co., Ltd., Tokyo, Japan) that had been prefilled with approximately 30 mL of water aerated with N_2 gas. The vials were then closed with butyl rubber stoppers. The gastight vials containing sediment samples were then transported to the laboratory and weighed. Prior to adding the sediment sample, the assembled vials, butyl rubber stoppers, and water were pre-weighed. After loading with sediment, the gas vials were shaken by hand for at least 3 min to establish equilibrium between the gas and water phases. The vials were flushed with nitrogen gas, forcing gaseous methane into a syringe that was connected to the rubber stopper with a tube. The syringe was left for 5 min to equilibrate the gas and the atmosphere, and the volume of gas was recorded. Methane was analyzed by gas chromatography (GC-8; Shimadzu, Kyoto, Japan). Methane concentrations were calculated as the mass of carbon in methane per mass of wet sediment (μg g^{-1}; CH_4-C wet $sediment^{-1}$). In addition, the difference in methane concentrations between sediment layers collected at 0–1 cm and 5–6 cm (ΔCH_4) was used to estimate the intensity of

234 biological methane oxidation. ΔCH_4 was calculated for each core sample.

235

236 *Data analysis*

237 The methane concentrations in the two sediment layers (0–1 cm and 5–6 cm) and ΔCH_4
238 were compared between the three sites and at different months using two-way ANOVA.
239 Post hoc analyses of differences among the three sites were conducted using Tukey's
240 HSD test. The methane concentration at Site C in December was not used for two-way
241 ANOVA because we did not survey at Sites A and B in December 2014. Linear models
242 were used to evaluate the influence of physicochemical conditions on $\delta^{13}C$ values in
243 larval chironomids. Although ΔCH_4 was considered a measure of methane production
244 and oxidation, ΔCH_4 depends strongly on methane concentrations in the 0–1 cm and
245 5–6 cm layers. DO above the lake bottom correlated with water temperature at all sites
246 ($P < 0.01$). Thus, we used ΔCH_4 and DO above the lake bottom as physicochemical
247 conditions in linear models. To avoid multicollinearity, we did not consider the
248 relationships between methane concentrations in the 0–1 cm and 5–6 cm layers and
249 water temperatures above the lake bottom. We used the statistical package R 3.5.0 (R
250 Development Core Team, 2017) for all of the statistical analyses.

251

252 **Results**

253 *Seasonal changes in water depth, temperature, and DO concentrations*

254 At Sites A and B, seasonal changes in water depth, temperature, and DO concentration
255 were measured from June 2014 to September 2015. The water depth was generally
256 shallow, fluctuating from 100 to 160 cm at Site A and from 100 to 170 cm at Site B
257 during June 2014 to August 2015. In September 2015, the water level was abnormally

high, reaching 205 cm, due to several days of heavy rain prior to sampling. In fact, monthly precipitation in September was clearly higher (349 mm) than that in other months (24 mm to 215 mm) (data from Japan Meteorological Agency, 2017, see supplementary material). Water temperatures at the surface and bottom of the lake tended to be slightly higher than the average monthly air temperature throughout the period of this study (Fig. 2). Differences in water temperature between the surface and bottom of the lake tended to be small at both sampling sites, but oxygen stratification often occurred (Fig. 2). Oxygen concentrations above the lake bottom were depleted at Sites A and B in summer and were as low as or less than ca. 2 mg l^{-1} at both sampling sites from July to August in 2014, at Site A from June to September in 2015, and at Site B from July to September in 2015 (Fig. 2). In particular, oxygen concentrations above the lake bottom decreased to as low or lower than ca. 1 mg l^{-1} at Site A in July (0.92 mg l^{-1}, 11.3% [saturation percentage]), August 2014 (0.13 mg l^{-1}, 1.6%), June (0.85 mg l^{-1}, 10.1%), July (0.47 mg l^{-1}, 5.6%), August 2015 (1.06 mg l^{-1}, 12.7%), at Site B in August 2014 (0.7 mg l^{-1}, 8.4%). From June 2007 to September 2008, water depth fluctuated from 110 to 175 cm at Site C with the exception of August 2008 (240 cm). The water column was relatively well mixed at Site C during this period (Fig. 2). Oxygen concentrations near the lake bottom were greater than 2 mg l^{-1} throughout this period. In August 2008, heavy rain (monthly precipitation was 296 mm, Japan Meteorological Agency, 2017) resulted in an exceptionally high water level (240 cm) and slight temporary stratification (Fig. 2). Consequently, oxygen concentrations above the lake bottom in August and September were lower (2.1 mg l^{-1}) than in other months (> 4 mg l^{-1}).

282 *Methane concentrations in sediment*

283 In areas covered by *N. nucifera* (Site A), methane concentrations in the 0–1 cm and 5–6
284 cm sediment layers peaked in September 2014 (0–1 cm: 3.8 ± 0.7 μg g^{-1} [CH_4-C wet
285 $sediment^{-1}$), 5–6 cm: 10.0 ± 1.2 μg g^{-1}) and in July 2015 (0–1 cm: 9.1 ± 4.2 μg g^{-1}, 5–6
286 cm: 14.7 ± 1.5 μg g^{-1}) (Fig. 3). In areas covered with *T. natans* (Site B), the methane
287 concentration in the surface sediment layer (0–1 cm) peaked in September in both 2014
288 (2.5 ± 1.9 μg g^{-1}) and 2015 (1.7 ± 0.4 μg g^{-1}), while the methane content of the
289 subsurface layer (5–6 cm) peaked in October 2014 (8.5 ± 2.4 μg g^{-1}) and in September
290 2015 (12.1 ± 5.9 μg g^{-1}). Methane concentrations tended to be higher in the 5–6 cm
291 layer than those in the 0–1 cm layer, indicating an increase in methane supply, and
292 higher methane oxidation rates, in the surface sediment. During winter and spring,
293 however, the methane concentrations in both the 0–1 cm and 5–6 cm layers remained
294 low. At Site A, the difference in methane concentration between the 0–1 cm and 5–6 cm
295 layers peaked in October 2014 (5.1 ± 1.8 μg g^{-1}) and in September 2015 (6.9 ± 4.8 μg
296 g^{-1}) when methane concentrations in both layers became high. At Site B, the difference
297 in methane concentration between layers peaked in October 2014 (7.4 ± 3.0 μg g^{-1}) and
298 in September 2015 (12.1 ± 5.9 μg g^{-1}). During the period from June 2007 to September
299 2008 at Site C, methane concentrations peaked in August 2007 (0–1 cm: 0.9 ± 0.6 μg g^{-1}
300 [CH_4-C wet $sediment^{-1}$], 5–6 cm: 6.5 ± 0.3 μg g^{-1}) and in September 2008 (0–1 cm: 2.9
301 ± 0.9 μg g^{-1} [CH_4-C wet $sediment^{-1}$], 5–6 cm: 8.7 ± 1.3 μg g^{-1}) (Fig. 3). During winter,
302 the methane concentrations in both the 5–6 cm and 0–1 cm layers remained low and no
303 methane was detected in the uppermost layer (0–1 cm) between October 2007 and
304 March 2008. Two-way ANOVA showed significant differences between sites in
305 methane concentrations in the 0–1 cm layer ($F_{2, 98} = 4.0$, $P < 0.001$). Post hoc Tukey's

306 HSD tests detected significant differences between Sites A and B ($P < 0.001$), and Sites
307 A and C ($P < 0.001$), whereas no significant differences were observed between Sites B
308 and C. For methane concentrations in the 5–6 cm layer, two-way ANOVA showed
309 significant differences between sites ($F_{2, 98} = 7.2$, $P < 0.01$). Post hoc Tukey's HSD tests
310 detected significant differences in methane concentrations in the 5–6 cm layers between
311 Sites A and C ($P < 0.01$), whereas no significant differences were observed between
312 Sites A and B, or between Sites B and C. There were no significant differences in ΔCH_4
313 between the 0–1 cm and 5–6 cm layers at all sites ($F_{2, 98} = 1.1$, $p > 0.05$).
314
315 *Stable carbon and nitrogen isotope ratios of larval chironomids and their potential food*
316 *sources*
317 The mean $\delta^{13}C$ levels in the sediment were –28.7 ± 0.2‰ (range: –29.1‰ to –28.2‰, *n*
318 = 27) at Site A and –28.7 ± 0.2‰ (–29.0‰ to –28.0‰, *n* = 30) at Site B. POM showed
319 a slightly higher depletion of $\delta^{13}C$ and a greater degree of fluctuation compared to the
320 sediment: –31.6 ± 2.7‰ (–35.7‰ to –27.5‰, *n* = 23) at Site A and –30.8 ± 2.3‰
321 (–35.2‰ to –27.7‰, *n* = 24) at Site B (Fig. 4). At Site C (data from Yasuno et al.
322 (2012)), the $\delta^{13}C$ level in the sediment was −27.6 ± 0.5‰ (range: −28.1‰ to −27.0‰, *n*
323 = 42) which was slightly higher than those at Sites A and B. The mean $\delta^{13}C$ levels of
324 POM at Site C was −31.3 ± 1.4‰ (range: −33.4‰ to −29.0‰, *n* = 42), similar to those
325 at Sites A and B. The mean $\delta^{15}N$ values in the sediment were 6.1 ± 1.0‰ (5.0‰ to
326 8.4‰, *n* = 27) at Site A and 5.6 ± 0.5‰ (4.5‰ to 6.8‰, *n* = 30) at Site B. The mean
327 $\delta^{15}N$ levels of POM were similar to those of sediment collected from the same site: 6.1
328 ± 1.9‰ (3.2‰ to 10.6‰, *n* = 27) at Site A and 5.2 ± 2.3‰ (1.2‰ to 8.3‰, *n* = 30) at
329 Site B. Since Yasuno et al. (2012) did not measure $\delta^{15}N$ levels, there are no data for

330 $\delta^{15}N$ at Site C. The $\delta^{13}C$ levels of larval *C. plumosus* showed wide inter-individual
331 variation during 2014 and 2015, ranging from −59.2‰ to −26.8‰ at Site A, and from
332 −57.9‰ to −24.7‰ at Site B (Fig. 5). Larval $\delta^{15}N$ also showed wide inter-individual
333 variation, ranging from 1.4‰ to 10.8‰ at Site A, and from –0.4‰ to 11.5‰ at Site B.
334 There were significant positive correlations between larval $\delta^{15}N$ and $\delta^{13}C$ at Site A (r^2 =
335 0.328, P< 0.001) and Site B (r^2 = 0.367, P < 0.001). From June to August at Sites A and
336 B in 2014 and 2015, the $\delta^{13}C$ levels of all larval individuals remained higher than −40‰.
337 At Site A, we were not able to collect any larvae in August 2014, August 2015, or
338 September 2015, despite taking more than 20 Ekman grab samples. In September 2015,
339 we were only able to collect one larva at Site B and deemed it insufficient for
340 determining a meaningful $\delta^{13}C$ levels. In October 2014, larval chironomids were
341 ^{13}C–depleted relative to those collected in August and September, and the $\delta^{13}C$ levels in
342 most individual larvae from both sites were lower than −40‰ in November 2014.
343 Individuals with lower $\delta^{13}C$ (< −40‰) were found even early the following spring
344 (March 2015). By May 2015, however, all larvae were ^{13}C-enriched, falling into a
345 narrow range of $\delta^{13}C$ levels: −30.9‰ to −28.1‰ at Site A, and −31.5‰ to −29.0‰ at
346 Site B. At Sites A and B, the $\delta^{13}C$ levels of all larval individuals remained higher than
347 −40‰ during June and July 2014. In August 2014, no larvae could be found at Site A,
348 despite taking more than 20 Ekman grab samples, whereas the $\delta^{13}C$ levels of larvae
349 were higher than −40‰ at Site B. In October 2014, larval chironomids were
350 ^{13}C–depleted relative to those collected in August and September, and the $\delta^{13}C$ levels of
351 most individual larvae from both sites were lower than −40‰ in November 2014. In
352 March 2015, some larval individuals remained ^{13}C–depleted (< −40‰), whereas others
353 were ^{13}C-enriched. The highest $\delta^{13}C$ levels in a single individual were −26.8‰ at Site A

354 and −24.7‰ at Site B. In May 2015, larvae showed higher $\delta^{13}C$ levels, with narrower
355 ranges, than in other months (Site A; −31.5 to −29.0‰, Site B; −30.9 to −28.1‰). In
356 July 2015, larvae collected from both Sites A and B remained ^{13}C-enriched (> −40‰).
357 In August 2015, no larvae were found at Site A, whereas the $\delta^{13}C$ levels of larvae were
358 higher than −40‰ at Site B. In September 2015, no larvae were collected at Sites A and
359 B. At Site C during 2007 and 2008, individual larval $\delta^{13}C$ levels ranged from −44.9‰ to
360 −26.7‰ (data from Yasuno et al. (2012)), which tended to be higher than those
361 collected from Sites A and B. Larval chironomids were ^{13}C–depleted in August 2007
362 and in September 2008 and most of these larvae showed $\delta^{13}C$ levels lower than −35‰.
363
364 *Relationships between $\delta^{13}C$ levels in larval chironomids and environmental factors*
365 Linear models indicated that larval $\delta^{13}C$ levels were negatively correlated with ΔCH_4 at
366 all three sites (Site A; $P < 0.01$, Site B; $P < 0.01$, Site C; $P < 0.001$, Table 2). At Sites A
367 and B, larval $\delta^{13}C$ levels were also negatively correlated with DO above the lake bottom
368 (Site A; $P < 0.01$, Site B; $P < 0.001$). In contrast, at Site C, larval $\delta^{13}C$ levels were not
369 significantly correlated with DO above the lake bottom.
370
371 **Discussion**
372 *Methane dynamics in sediments*
373 Low oxygen conditions above a lake bottom can enhance methane cycles (*i.e.*, methane
374 production and oxidation) in the sediment (Eller et al., 2005; Deines et al., 2007b;
375 Gentzel et al., 2012). Macrophytes, particularly floating-leaved and emergent plants,
376 can prevent water turbulence and gas exchange between the lake surface and the air
377 (Frodge et al., 1990; Caraco et al., 2006). Simultaneously, oxygen may be actively

consumed during the microbial decomposition of dead macrophyte deposits, resulting in the depletion of DO at the lake bottom (Turner et al., 2010; Yamaki & Yamamuro, 2013; Kato et al., 2016). Thus, macrophytes such as lotus and *T. natans* may promote methane cycles in the sediment. Before the expansion of lotus vegetation in Lake Izunuma (from 2007 to 2008, Site C), the water column was frequently well mixed and DO was sufficiently supplied to the lake bottom nearly throughout the year. In one exceptional event, oxygen concentrations near the lake bottom were depleted to approximately 2 mg l^{-1} due to temporal stratification caused by a sudden increase in water level after a heavy rain in August 2008 (Fig. 2). After the lotus expanded to cover more than 80% of the water surface, DO concentrations near the lake bottom were consistently depleted to < 1 mg l^{-1} within macrophyte-covered areas (Sites A and B) during summer (Fig. 2). In addition, DO concentrations at the lake bottom were significantly lower at Sites A and B (macrophyte-covered areas) than at Site C (open water) ($P < 0.01$). Therefore, water surface coverage by lotus and *T. natans* can result in DO depletion. Methane concentrations in sediment layers collected at 0–1 cm and 5–6 cm were significantly higher at Site A, which was covered with lotus vegetation, than at Site C which had no vegetation (0–1 cm; $P < 0.001$, 5–6 cm; $P < 0.01$, Fig. 3). Since low oxygen conditions in overlying water can promote methane production in the sediment (Eller et al., 2005; Deines et al., 2007b; Gentzel et al., 2012), strong oxygen depletion near the lake bottom at Site A may lead to high methane concentrations during July and September. In July 2015, when oxygen concentrations were lower than 1 mg l^{-1}, methane concentrations in both the 0–1 cm and 5–6 cm layers were the highest encountered in this study period. Because strong oxygen depletion was also observed in June 2015, low oxygen conditions may continue for a relatively long period, stimulating methane production

and accumulation of methane in the sediment. Seasonal inputs of organic matter to the lake bottom also stimulate biological methane production in surface sediments (Chan et al., 2005; Schwarz et al., 2008). Every autumn, the lotus plant withers and organic matter derived from the macrophytes accumulates on the lake bottom. Fujibayashi et al. (2013) analyzed the fatty acid composition of sediments collected from Lake Izunuma after the lotus expansion and found that sediment organic matter was derived primarily from lotus. Therefore, seasonal inputs of organic matter from lotus may lead to a greater accumulation of methane in the sediment at Site A than at Site C. Conversely, methane concentrations in the 0–1 cm and 5–6 cm layers at Site B were not significantly different than those at Site C. The effects of macrophytes on DO levels depend on morphological (*e.g.*, floating-leaved, submersed or emergent plants) and structural differences such as stem density and leaf size (Caraco et al., 2006; Bunch et al., 2010). Because lotus produces much larger leaves than *T. natans*, the input of organic matter to the sediment is likely larger in areas covered with lotus vegetation than in those dominated by *T. natans* vegetation. Therefore, methane production was lower at Site B than at Site A. Oxygen generally penetrates into sediment from the overlying water, leading to methane oxidation at sediment surface (c.a. < 1cm depth, Sobek et al., 2009; Gentzel et al., 2012). Gentzel et al. (2012) investigated vertical distributions of MOB DNA in lake sediment and showed a maximum concentration at 1 mm sediment depth. Consequently, a steep gradient of methane concentration was observed over several centimeters into the sediment. Hence, we considered ΔCH_4 (difference in methane concentrations between 0–1 cm and 5–6 cm layers) as an indicator of methane oxidation. In contrast to absolute methane concentrations, there was no significant difference in ΔCH_4 among sites. At macrophyte-rich Sites A and B, ΔCH_4 peaked in September or

October. At Site C, which was not covered in vegetation, ΔCH_4 peaked in August or September when methane concentrations in the sediment were high. ΔCH_4 maintained values as high as or higher than c.a. 3 μg g^{-1} (CH_4-C wet $sediment^{-1}$) at Sites A and B even when DO concentrations were less than 1 mg l^{-1} (*e.g.*, August 2014 and July 2015), indicating methane oxidation under low oxygen conditions. In fact, MOB are tolerant to hypoxic conditions (Gentzel et al., 2012). In November, ΔCH_4 levels at Sites A and B were higher than that at Site C, probably because of greater methane accumulation in the sediments at Site A and B than due to organic matter input from macrophytes. Therefore, larger amounts of MOB were able to inhabit the surface sediment during June or July to November.

Assimilation of MDC by larval chironomids

We observed marked depletion in $\delta^{13}C$ levels in larval chironomids in autumn at both Site A (lotus vegetation) and Site B (*T. natans* vegetation), and in late summer at Site C (with no vegetation). The $\delta^{13}C$ level of larval individuals reached –59.2‰ at Site A, –57.9‰ at Site B and –44.9‰ at Site C. Although consumers with depleted $\delta^{13}C$ (< –40‰) are typically considered to have assimilated MDC by foraging on MOB, it is possible that heterotrophically respired carbon, which is often abundant in eutrophic bodies of water, may provide an alternative ^{13}C-depleted carbon source (Lennon et al., 2006). Foraging algal material that incorporates respired carbon may lead to depleted $\delta^{13}C$ signatures in consumers. In this study, however, we found significant positive correlations between larval $\delta^{15}N$ and $\delta^{13}C$ (Fig. 4). Although Yasuno et al. (2012) did not measure $\delta^{15}N$ values in larval chironomids collected during 2007 and 2008, larvae collected in 2006 showed a similar correlation between $\delta^{15}N$ and $\delta^{13}C$ (Yasuno et al.,

2013). These correlations indicate that ^{13}C-depleted larval chironomids used MOB as a food source. *C. plumosus* live in tubes constructed from silk and sediment, which are irrigated during feeding and respiration (McLachlan, 1977; Yasuno et al., 2013). MOB are more abundant on the inner wall of the robust U-shaped larval tube than in surface sediment (Kajan & Frenzel, 1999; Gentzel et al., 2012). Tube-dwelling chironomid larvae excrete ammonium within their tubes (Fukuhara & Yasuda, 1989; Devine & Vanni, 2002). The microbial community within the tube, including MOB, assimilates this abundant ammonium as a nitrogen source, resulting in negative $\delta^{15}N$ signatures (Macko et al., 1987). Therefore, ^{13}C-depleted chironomid larvae could assimilate MDC by ingestion of MOB. Large inter-individual $\delta^{13}C$ variability was found in larvae collected at all sites, even among individuals collected at the same time. In particular, the ranges of $\delta^{13}C$ in larvae collected in March 2015 were 27.2‰ at Site A and 33.2‰ at Site B (Fig. 5). Similar inter-individual variability has often been reported when chironomid larvae were ^{13}C–depleted due to the assimilation of MDC (Grey et al., 2004a; Deines et al., 2007b; Ravinet et al., 2010). The observed inter-individual variability seemed to reflect differences in reliance on MOB among individuals. Larval *C. plumosus* are known to switch their feeding behavior (filter feeding or deposit feeding) (McLachlan, 1977) and Deines et al. (2007a) showed experimentally, using ^{13}C-labeled methane, that this can explain inter-individual variability. Therefore, the isotopic inter-individual variability observed herein may reflect differences in feeding behavior among larval individuals.

Seasonality of use of MDC by larval chironomids

Before the lotus expansion, $\delta^{13}C$ levels of larval chironomids decreased at Site C (no

474 vegetation) during late summer or early autumn (Fig. 5) when methane concentrations
475 in the sediment peaked (Fig. 3), indicating an increase in larval reliance on MDC.
476 However, after the lotus expansion, no larvae were collected at Site A during August or
477 September, and at Site B in September. DO was strongly depleted within
478 macrophyte-covered areas during June or July to August (c.a. < 1 mg l^{-1}, Fig. 2). Thus,
479 macrophyte coverage may make the lake bottom too harsh an environment for larval
480 chironomids. In fact, the density of benthic invertebrates, including larval *C. plumosus*,
481 was extremely low at Sites A and B during August and September (Yasuno et al*., 2*015).
482 Although larvae were collected at Site B in August 2014 and 2015, they were not
483 ^{13}C-depleted (> –40‰) (Fig. 5), indicating less assimilation of MDC by larval
484 chironomids. The activity of MOB depends on the availability of both oxygen and
485 methane (Borrel et al., 2011). However, MOB are more likely to be found in surface
486 sediments with hypoxic overlying water (<1 mg l^{-1}). This is likely due to the large
487 methane supply from the sediment (Gentzel et al., 2012). ΔCH_4 usually peaked in
488 September at Sites A and B, indicating high methane oxidation rates and large amounts
489 of MOB biomass (Fig. 3). *Chironomus anthracinus* Zetterstedt larvae, which are
490 tolerant to low oxygen conditions mush like *C. plumosus*, are aestivate in oxygen levels
491 less than 0.5 mg l^{-1} (Hamburger et al., 1994). Therefore, extreme hypoxia could make
492 larval chironomids inactive and prevent them from feeding on MOB. Consequently,
493 these larvae were not ^{13}C-depleted. During October and November 2014, larval
494 chironomids from both Sites A and B became markedly ^{13}C–depleted. Similar isotopic
495 depletion in autumn has been reported in temperate dimictic lakes, likely because the
496 renewed availability of oxygen at the sediment surface can stimulate the production of
497 MOB, thereby increasing the importance of MOB in the diet of larval chironomids

498 (Grey et al., 2004b; Deines et al., 2007b). In Lake Izunuma, oxygen is supplied to the
499 oxic-anoxic interface (lake bottom) when the lotus starts to wither in October. This may
500 stimulate MOB and facilitate the entry of MDC into the food web. In October 2014,
501 methane concentrations in the 0–1 cm and 5–6 cm layers collected at Sites A and B
502 were clearly higher than that at Site C (Fig. 3). Organic matter derived from dead lotus
503 is supplied to the lake sediment during autumn. Microbial decomposition of organic
504 matter by fermentative bacteria produces substrate material, such as H_2 and acetate, for
505 methanogenesis. This promotes biogenic methane production (Borrel et al., 2011).
506 Therefore, methane production and oxidation may be stimulated in autumn, thereby
507 increasing the availability of MOB to larval chironomids within macrophyte-covered
508 areas. Relatively large numbers of larvae were easily collected at Sites A and B in
509 October 2014. Site A did not yield any larvae during August and September, and Site B
510 did not yield larvae in September. These results indicate the emergence of adult
511 chironomids and the recruitment of larvae during September and October. The
512 emergence of *C. plumosus* is known to occur two or three times per year in Japan, with
513 a latter emergence often occurring in autumn (Nakazato & Hirabayashi, 1998). The
514 density of fourth-instar *C. plumosus* larvae increased from September to October
515 (Yasuno et al., 2009), indicating emergence and recruitment of larvae. Aquatic insects
516 typically have fast turnover rates. Hamilton et al. (2004) showed that aquatic insects in
517 streams have $\delta^{15}N$ half-lives (the time required for a 50% change in isotope ratio
518 following a switch in food source) shorter than 12 days. Doi et al. (2007) showed that
519 the $\delta^{13}C$ and $\delta^{15}N$ half-lives of fourth-instar larvae of *Chironomus acerbiphilus*
520 Tokunaga after molting were approximately 6 days. Therefore, low $\delta^{13}C$ levels in larval
521 *C. plumosus* in October 2014 may reflect their feeding history over a relatively short

term (after recruitment). Some larval chironomids collected at Sites A and B in March 2015 also showed extremely low $\delta^{13}C$ levels (Fig. 5). However, methane concentrations in the sediment during this time were almost at their lowest (Fig. 3), indicating that a relatively small biomass of MOB was available to larval chironomids. Larval *C. plumosus* is known to go inactive and rarely feeds at temperatures below 5°C (Hilsenhoff, 1966). Although we did not measure water temperature during December 2014 and February 2015, average air temperatures during that period ranged from 0.3 to 1.2°C (Fig. 2). Thus, the low $\delta^{13}C$ levels measured in larvae in March 2015 may reflect MOB ingestion during the previous autumn because of low larval feeding activity during winter. However, larval $\delta^{13}C$ tended to be higher in March 2015 than in November 2014 at both Sites A and B (Fig. 5). In March 2015, water temperature rose above 5°C (Fig. 2), where larval *C. plumosus* begin filter-feeding (Hilsenhoff, 1966). These larvae could start to feed on POM or sediment organic matter in March 2015, resulting in higher larval $\delta^{13}C$ levels than those measured the previous autumn. ^{13}C–depleted larval chironomids (< –40‰) disappeared and all larvae fell into a narrow range of $\delta^{13}C$ levels in May 2015 at Sites A and B (Fig. 5). In Lake Izunuma, the emergence of adult *C. plumosus* was observed during April and early May (Yasuno N. personal observation). The emergence of overwintered, ^{13}C–depleted larvae could result in an increase in larval $\delta^{13}C$.

Effects of macrophyte coverage on use of MDC by larval chironomids

We showed that macrophyte vegetation can greatly affect the trophic transfer of MDC to benthic larval chironomids, and can also affect the seasonality of larval $\delta^{13}C$ levels. During summer, hypoxia associated with macrophyte vegetation may make the lake

546 bottom too harsh for larval chironomids, or render them inactive, thereby limiting the
547 trophic transfer of MDC to benthic consumers. In contrast, autumnal oxygen supply to
548 the lake bottom may stimulate MOB activity and the feeding activity of chironomid
549 larvae. In addition, the accumulation of organic matter from dead macrophytes may
550 promote methane production and oxidation during autumn, resulting in enhanced
551 trophic transfer of MDC to chironomid larvae. The trophic transfer of MDC to benthic
552 consumers can be affected by the supply of methane or oxygen to MOB (Grey et al.,
553 2004b; Deines et al., 2007b; Yasuno et al., 2012). In open areas (lacking vegetation) of
554 shallow (polymictic) lakes, there may be a constant supply of DO to the MOB habitat at
555 the lake bottom. Consequently, the use of MDC may be strongly affected by the
556 availability of methane (Yasuno et al., 2012). In contrast, in dimictic lakes, hypolimnetic
557 hypoxia could render MOB at the surface sediment and larval chironomids inactive and
558 limit the MDC pathway to benthic consumers during the summer stratification period
559 (Grey et al., 2004b). Our findings show that the trophic transfer of the MDC pathway
560 during late summer and early autumn in a shallow lake may be greatly affected by the
561 extreme hypoxia associated with floating-leaved and emergent macrophyte vegetation,
562 causing seasonal patterns in the use of MDC by larvae that more closely resemble those
563 in dimictic lakes than that of Lake Izunuma before the lotus expansion (Yasuno et al.,
564 2012). Agasild et al. (2014) also reported autumnal ^{13}C depletion in larval chironomids
565 from a plant-dominated site (submerged and floating-leaved plants) in Lake Võrtsjärv in
566 Estonia. Larvae exhibited relatively high $\delta^{13}C$ levels in September with significantly
567 lower levels in November. Thus, similar autumnal ^{13}C-depletion in larval chironomids
568 may occur among vegetated areas in other lakes or ponds. Coverage with
569 floating-leaved and emergent macrophyte vegetation is a common environment in

570 shallow water bodies such as the littoral zones of lakes and small ponds. Therefore,
571 although further studies are necessary, our findings represent an important contribution
572 to the understanding of the carbon cycle in wetlands.
573
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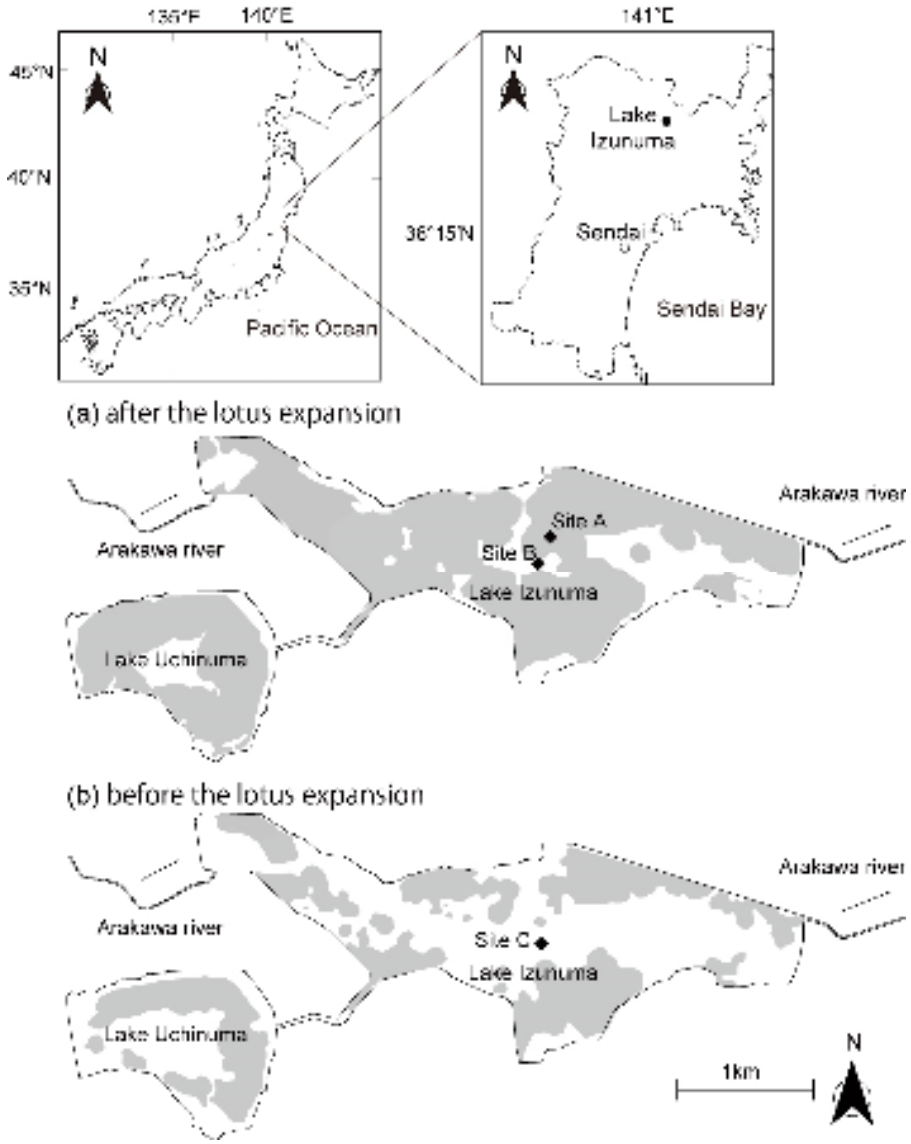
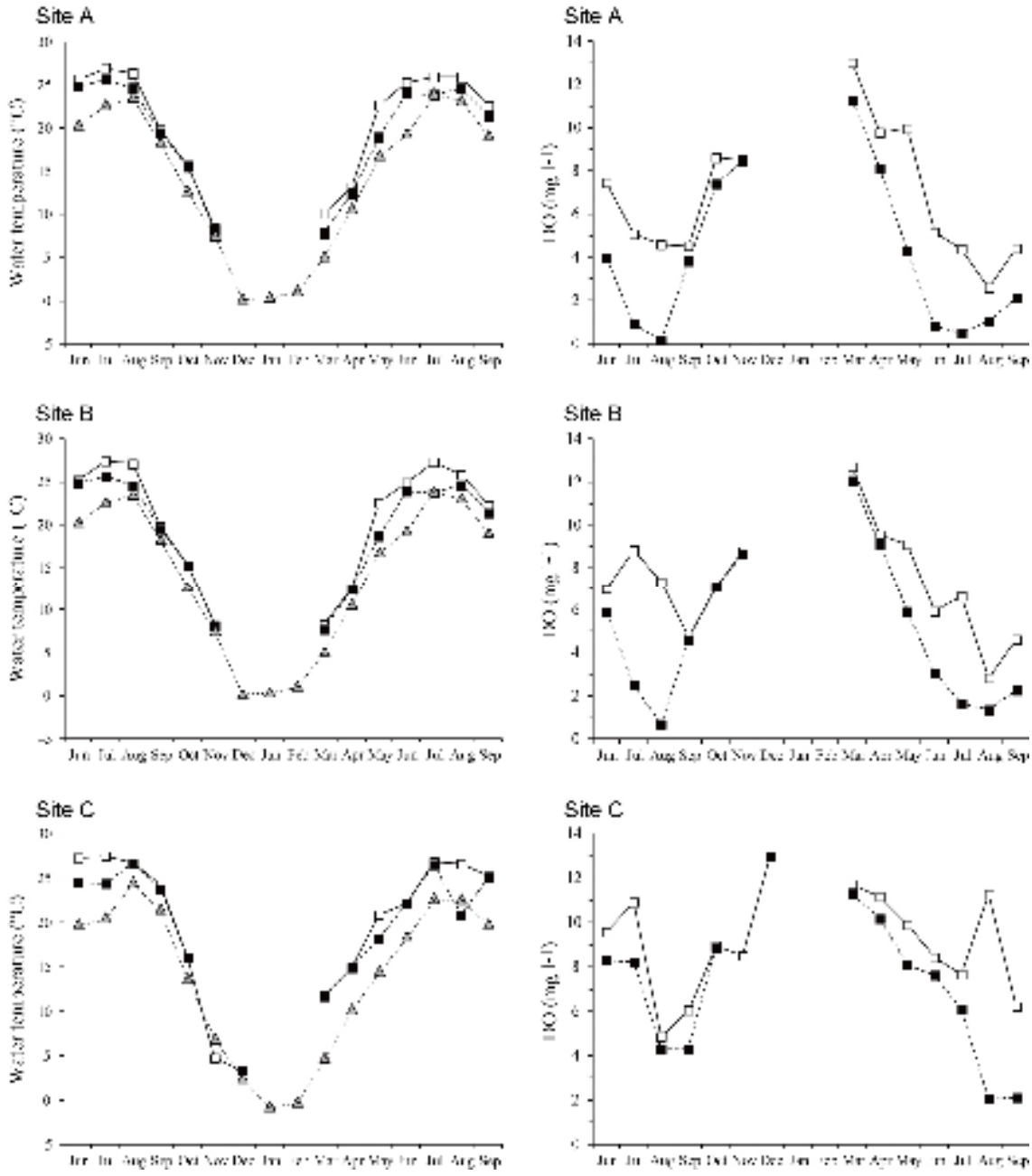


Fig. 1. Sampling locations in Lake Izunuma. The gray area represents the distribution of lotus *Nelumbo nucifera*. (a) The distribution of lotus after the expansion was drawn based on a photograph taken in August 2012 (7th Izunuma-Uchinuma Nature Restoration Committee, 2013). Sites A and B were among the lotus vegetation and *Trapa natans* vegetation, respectively. (b) The distribution of lotus before its expansion was drawn based on ALOS-PALSAR images of Lake Izunuma and Uchinuma in August 2007 (© METI, JAXA). Site C (with no vegetation) was a sampling point used in a previous study (Yasuno et al., 2012).



860

861 Fig. 2. Seasonal variation in water temperature and dissolved oxygen concentrations.
862 Open symbols show data recorded at the water surface; solid symbols indicate data
863 recorded 10–30 cm above the lake bottom. Gray triangles show average monthly air
864 temperature (data from Japan Meteorological Agency (2017)).

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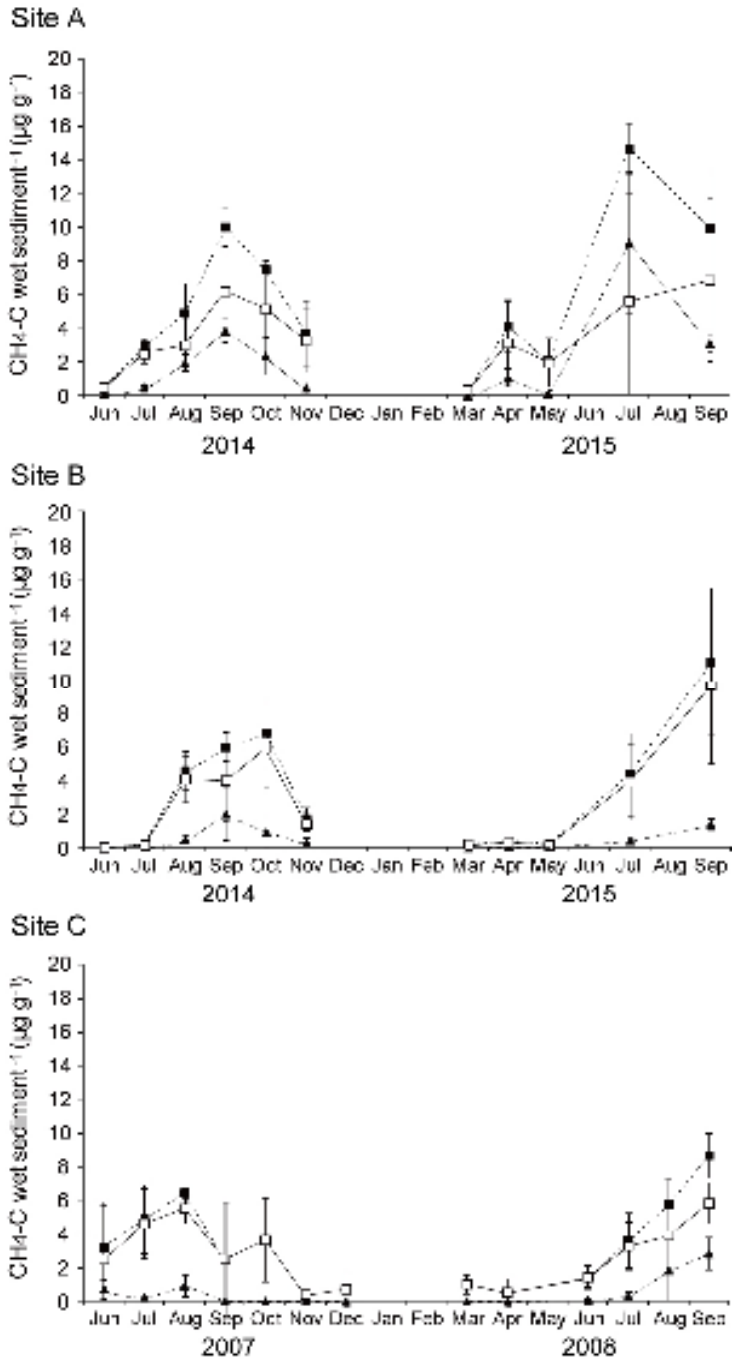
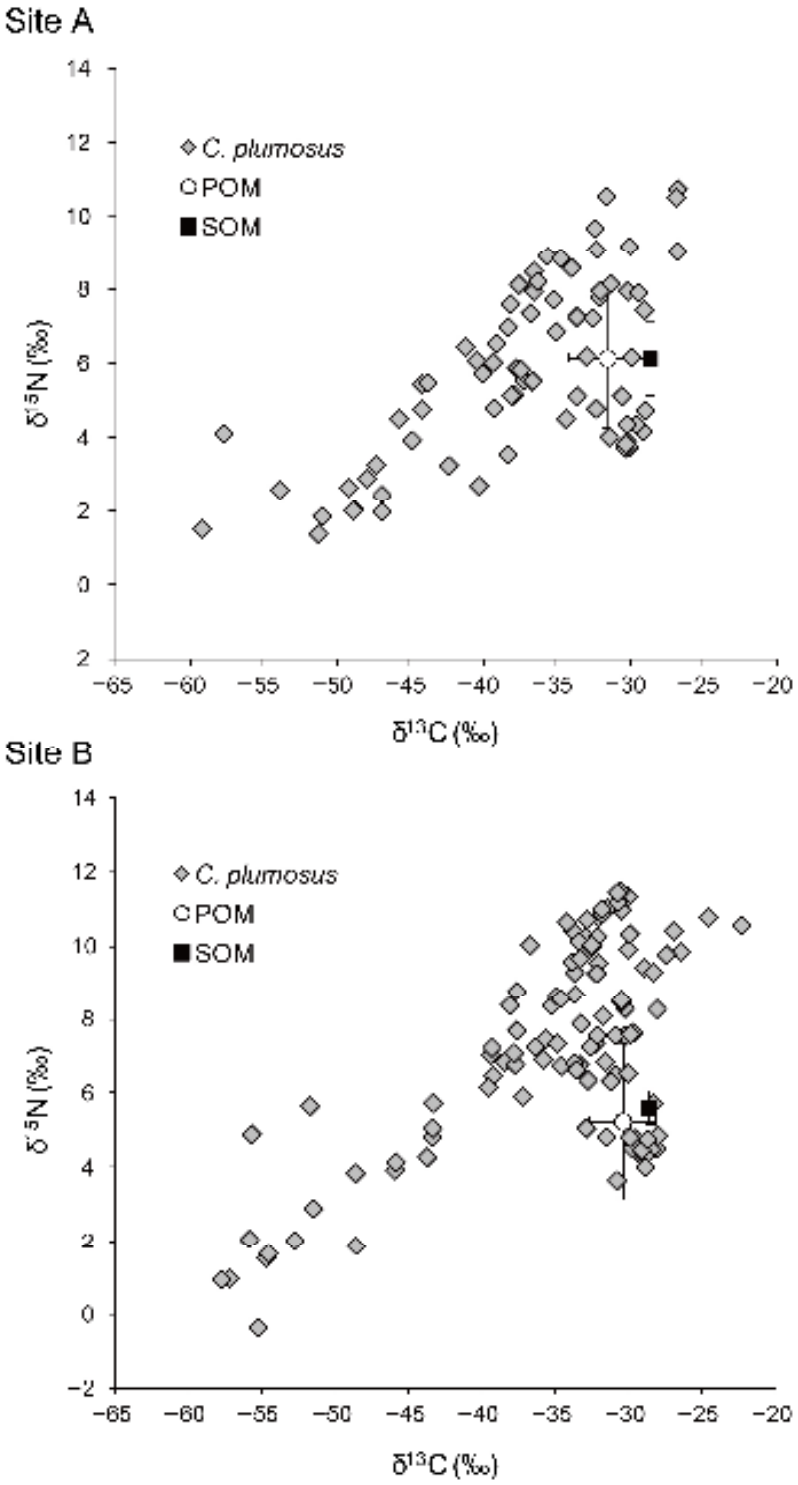


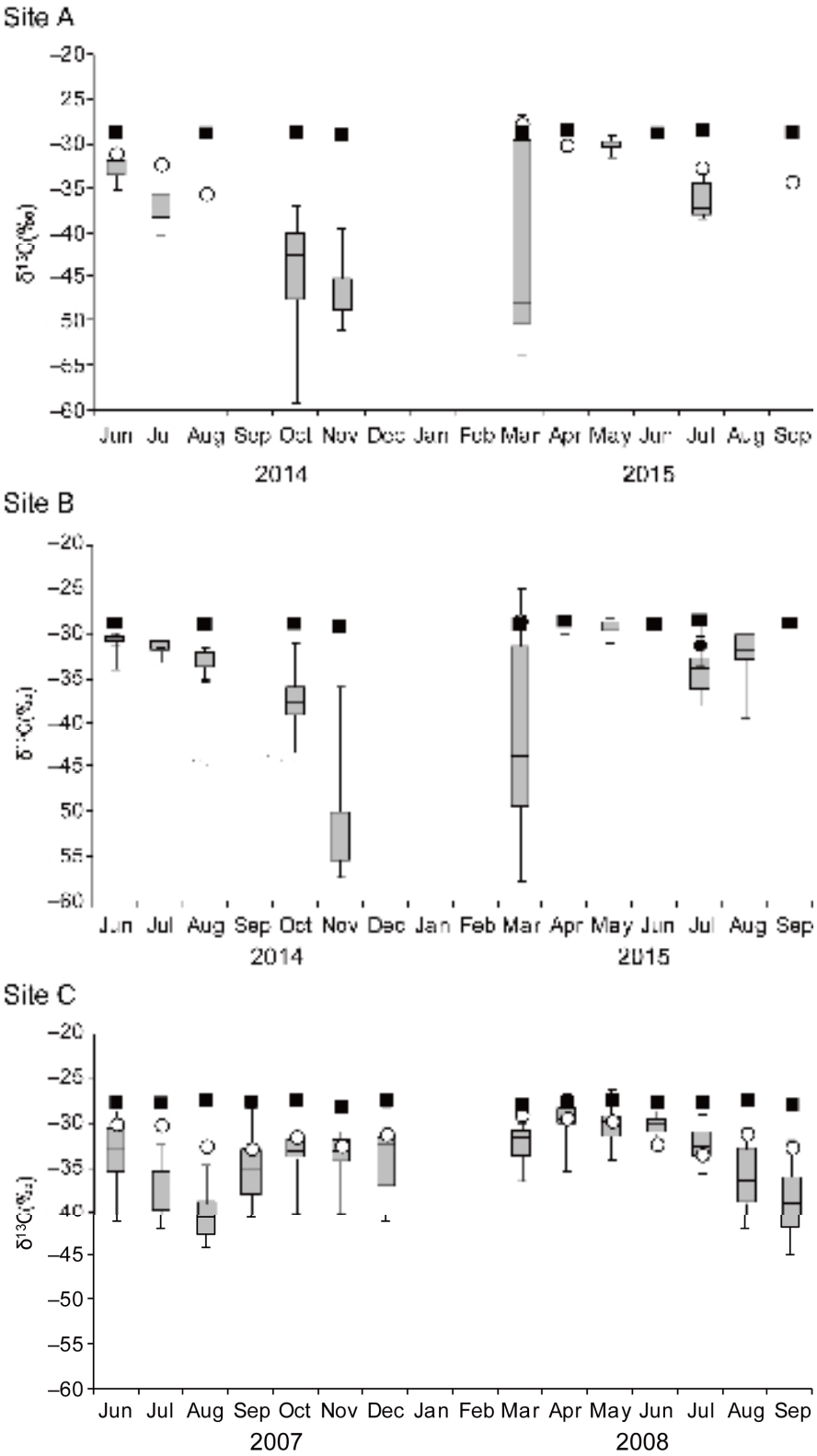
867

868 Fig. 3. Seasonal variations in methane concentration in two sediment layers at three
869 sites. Symbols represent mean concentrations and error bars indicate standard deviations.
870 Closed triangles and squares represent methane concentrations at sediment depths of
871 0–1 cm and 5–6 cm, respectively. Open squares represent differences in methane
872 concentrations between 0–1 cm and 5–6 cm layers (ΔCH_4).



873

874 Fig. 4. Interindividual isotopic variations of *Chironomus plumosus* larvae collected
875 during the study period from among lotus vegetation (Site A) and *Trapa* vegetation (Site
876 B). Error bars represent standard deviations of all samples of POM and sediment.



877

878 Fig. 5. Seasonal variation in stable carbon isotope ratios of larval *Chironomus plumosus*

879 and their potential food sources collected during 2014–2015 from Sites A and B, and

during 2007–2008 from Site C. Boxes, open circles and closed squares represent stable carbon isotope ratios of larval *C. plumosus*, POM and sediment, respectively. No larvae were collected from Site A in August 2014, August 2015, or September 2015. We did not measure the stable isotope ratio of larval chironomids from Site B in September 2015 since only one individual was collected.

886 Table 1. Sample list for stable isotope analyses.

Sampling month	*Chironomus plumosus*		POM		sediment	
	Site A	Site B	Site A	Site B	Site A	Site B
Jun 2014	7	7	3	3	3	3
Jul 2014	10	8	3	3		
Aug 2014		16	3	3	3	3
Oct 2014	8	11			3	3
Nov 2014	8	11			3	3
Mar 2015	7	7	3	3	3	3
Apr 2015			3	3	3	3
May 2015	10	13	2	3		
Jun 2015					3	3
Jul 2015	9	16	3	3	3	3
Aug 2015		5				
Sep 2015			3	3	3	3

Sampling month	*Chironomus plumosus*	POM	sediment
	Site C	Site C	Site C
Jun 2007	14	3	3
Jul 2007	14	3	3
Aug 2007	13	3	3
Sep 2007	19	3	3
Oct 2007	23	3	3
Nov 2007	20	3	3
Dec 2007	19	3	3
Mar 2008	19	3	3
Apr 2008	20	3	3
May 2008	20	3	3
Jun 2008	20	3	3
Jul 2008	20	3	3
Aug 2008	20	3	3
Sep 2008	19	3	3

*Samples collected at Site C were from Yasuno et al. (2012)

887

888

889 Table 2. Parameter estimates and standard error of linear models to explain the $\delta^{13}C$

890 levels in larval chironomids by physicochemical factors.

Model	Parameter	Estimate	SE	*t* value	
Site A					
	(Intercept)	-28.730	2.628	-10.935	* * *
	ΔCH_4	-1.727	0.530	-3.257	* *
	DO	-0.814	0.261	-3.117	* *
Site B					
	(Intercept)	-26.897	1.797	-14.966	* * *
	ΔCH_4	-0.786	0.293	-2.680	* *
	DO	-1.355	0.224	-6.041	* * *
Site C					
	(Intercept)	-28.980	1.230	-23.569	* * *
	ΔCH_4	-1.603	0.197	-8.143	* * *
	DO	-0.090	0.100	-0.899	

SE shows standard error.

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

891

892

893 Supplementary material

year	month	Monthly precipitation (mm)		Average monthly air temperature (°C)	
		2007/2008	2014/2015	2007/2008	2014/2015
2007/2014	Jun	134	140	19.9	20.4
	Jul	196	118	20.6	22.7
	Aug	84	135	24.6	23.6
	Sep	137	83	21.6	18.4
	Oct	107	215	13.8	12.8
	Nov	32	79	7	7.6
	Dec	54	127	2.6	0.3
2008/2015	Jan	18	28	−0.6	0.5
	Feb	24	24	−0.1	1.2
	Mar	42	146	4.9	5.2
	Apr	75	97	10.3	10.7
	May	109	48	14.6	16.9
	Jun	71	134	18.5	19.4
	Jul	141	67	22.8	24
	Aug	296	146	22.7	23.2
	Sep	79	349	19.9	19.2

894