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Chlorophyll *a* specific $\Delta^{14}C$, $\delta^{13}C$ and $\delta^{15}N$ values in stream periphyton: implications for aquatic food web studies

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Abstract

We determined the isotopic composition of chlorophyll *a* in periphytic algae attached to a streambed substrate (periphyton). The samples were collected from a stream flowing on limestone bedrock in the Seri River, central Japan. Stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) and natural radiocarbon abundances ($\Delta^{14}C$) were measured in chlorophyll *a* ($\delta^{13}C_{chl}$, $\delta^{15}N_{chl}$ and $\Delta^{14}C_{chl}$) and bulk ($\delta^{13}C_{bulk}$, $\delta^{15}N_{bulk}$ and $\Delta^{14}C_{bulk}$) for periphyton, pure aquatic primary producer (*Cladophora* sp.) and terrestrial primary producer (*Quercus glauca*). Periphyton $\delta^{13}C_{bulk}$ and $\delta^{13}C_{chl}$ values did not necessarily correspond to $\delta^{13}C_{bulk}$ for an algal-grazing specialist (Mayfly larva, *Epeorus latifolium*), suggesting that periphyton $\delta^{13}C$ values do not faithfully trace carbon transfer between primary producers and primary consumers. Periphyton $\Delta^{14}C_{chl}$ values (−258 ‰ in April and −190 ‰ in October) were slightly lower than $\Delta^{14}C_{bulk}$ values (−228 ‰ in April and −179 ‰ in October), but were close to the $\Delta^{14}C$ value for dissolved inorganic carbon (DIC) (−217 ± 31 ‰), which is a mixture of weathered carbonates ($\Delta^{14}C = -1000$ ‰) and dissolved atmospheric CO_2 ($\Delta^{14}C$ approximately +30 ‰ in 2013). $\Delta^{14}C_{chl}$ values were also close to $\Delta^{14}C_{bulk}$ for *E. latifolium* (−215 ‰ in April and −199 ‰ in October) and *Cladophora* sp. (−210 ‰), whereas the $\Delta^{14}C_{bulk}$ value for *Q. glauca* (+27 ‰) was closer to $\Delta^{14}C$ for atmospheric CO_2. Although the bulk isotopic composition of periphyton is recognised as a surrogate for the photosynthetic algal community, natural periphyton is a mixture of aquatic and terrestrial organic materials. Our results indicate that the bulk periphyton matrix at the study site consists of 89 to 95 % algal carbon (derived from ^{14}C-depleted DIC) and 5 to 11 % terrestrial organic carbon (derived from ^{14}C-enriched atmospheric CO_2).

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1 Introduction

The bioavailable energy in a natural ecosystem often originates not only from in situ photoautotrophs, but also from resources produced in other ecosystems. In most freshwater ecosystems (e.g., streams), periphytic algae attached to a substrate (periphyton) play an important role as benthic primary producers (Allan and Castillo, 2007). Terrestrial material (e.g., leaf detritus) is another resource for animals, especially in small headwater streams (Vannote et al., 1980). Although the relative importance of aquatic and terrestrial resources for food webs is a major concern in stream ecology (Vannote et al., 1980; Junk et al., 1989; Thorp and Delong, 1994), the energy flow from periphyton to animal consumers has not yet been adequately assessed, because few studies have traced algal signatures through trophic pathways. In stream food webs, macroinvertebrates are the dominant animal consumers, and observation of their gut contents is a direct measure that can be used to trace energy flow (Winemiller, 1990; Hall et al., 2000). However, the diets of stream macroinvertebrates are sometimes too diverse to identify, and are not necessarily identical to what they actually assimilate (Whitledge and Rabeni, 1997; Finlay, 2001).

The stable isotope ratios of carbon (δ^{13}C) and nitrogen (δ^{15}N) have contributed to food web research over the last 40 years (after DeNiro and Epstein, 1978; Minagawa and Wada, 1984). In stream ecosystems, environmental heterogeneity within a small area (e.g., habitat variability in terms of light or flow regimes) is reflected in variations in periphyton δ^{13}C (Ishikawa et al., 2012a), which often makes it difficult to estimate the relative importance of aquatic (e.g., periphyton) and terrestrial (e.g., leaf detritus) resources for macroinvertebrates (Finlay et al., 1999; Zah et al., 2001; Doi et al., 2007; Dekar et al., 2009).

Recently, periphyton and terrestrial leaf detritus have been distinguished using natural radiocarbon abundances (Δ^{14}C). Periphyton Δ^{14}C is derived from aged carbon reservoirs, such as bedrocks and soils, and is relatively low compared to terrestrial leaf detritus that reflects Δ^{14}C value for modern atmospheric CO_2. Macroinvertebrate and fish $\Delta^{14}C$ values lie between those for periphyton and leaf detritus, indicating that $\Delta^{14}C$ can be used to estimate the energy flow in stream food webs (Ishikawa et al., 2014b). Although bulk $\delta^{13}C$, $\delta^{15}N$ and $\Delta^{14}C$ values imply that the periphyton is isotopically identical to periphytic algae, it is actually a mixture of algae, heterotrophic fungi and bacteria, together with the exopolymeric substances exuded by these organisms, protozoa, small metazoa and other non-living particulate organic materials (Cross et al., 2005). All of these components may originate from different sources and have unique $\delta^{13}C$, $\delta^{15}N$ and $\Delta^{14}C$ values (Hladyz et al., 2011; Ishikawa et al., 2012b; Imberger et al., 2014; Fellman et al., 2015). Therefore, the algal and non-algal taxonomic compositions of the periphyton community potentially influence its bulk isotopic composition.

Because the densities of living algae and non-algal materials (e.g., leaf detritus or animal remains) usually differ, algae and other materials in periphyton are sometimes separated by centrifuging slurry washed from stream cobbles or rocks (Hamilton and Lewis, 1992; Small et al., 2011). However, the density-separation method does not often work well when the non-algal fraction contains large amounts of dead algae, and these two components are barely distinguishable even under a microscope (Finlay, 2004). The $\delta^{13}C$ and $\Delta^{14}C$ values for bulk periphyton and its potential carbon sources (e.g., particulate organic carbon: POC, dissolved organic carbon: DOC and dissolved inorganic carbon: DIC) can be used to separate the algal carbon fraction from the non-algal carbon fraction (Fellman et al., 2015), although it is still difficult to quantitatively and directly estimate the relative abundances of the aquatic (i.e., algae) and terrestrial (i.e., leaf detritus) carbon fractions in periphyton based on their bulk isotopic compositions.

To assess the accuracy of using bulk isotopic composition of periphyton to represent that of aquatic primary producers, we used an algal biomarker found in the periphyton matrix. Chlorophylls are the ubiquitous antenna pigments of the photoautotrophs, and the chlorophyll *a* concentration, in particular, has been used as an indicator of in situ primary production because it is immediately degraded in the inactive state (Carpenter et al., 1986; Amir-Shapira et al., 1987). Several previous studies have successfully used the δ^{13}C, δ^{15}N and Δ^{14}C values for chlorophyll *a* and its derivatives to understand modern environments or reconstruct palaeoenvironments (e.g., Hayes et al., 1987; Sachs et al., 1999; Ohkouchi et al., 2005, 2008; Kusch et al., 2010; Tyler et al., 2010; Higgins et al., 2012).

In this study, differences in the δ^{13}C, δ^{15}N and Δ^{14}C values in chlorophyll *a* ($\delta^{13}C_{chl}$, $\delta^{15}N_{chl}$ and $\Delta^{14}C_{chl}$) and bulk ($\delta^{13}C_{bulk}$, $\delta^{15}N_{bulk}$ and $\Delta^{14}C_{bulk}$) for periphyton were compared to distinguish aquatic (i.e., algae) and terrestrial (i.e., leaf detritus) carbon fractions in the periphyton community. Because the Δ^{14}C value is internally corrected by its δ^{13}C (Stuiver and Polach, 1977), $\Delta^{14}C_{chl}$ does not depend on the isotopic fractionation during algal photosynthesis and chlorophyll *a* biosynthesis. Therefore, the $\Delta^{14}C_{chl}$ value for periphyton should reflect that for photosynthetic autotrophs (i.e., primary producers) and can be used as a proxy of aquatic carbon for animals at higher trophic levels of the food web. The $\Delta^{14}C_{chl}$ values for periphyton, DIC and an algal-grazing specialist were compared to identify the trophic transfers of carbon. Pure primary producers (i.e., aquatic algae and terrestrial plants) were used to assess the potential differences in δ^{13}C, δ^{15}N and Δ^{14}C values between chlorophyll *a* and bulk cells.

2 Materials and methods

2.1 Study site and sample collection

In April and October 2013, field samplings were undertaken at Kawachi in the upland of the Seri River (watershed area = 30 km^2, 35°15′ N, 136°20′ E in Shiga Prefecture, central Japan), which flows into Lake Biwa, the largest lake in Japan. The reach of the river studied flows over limestone–basalt bedrock (dominated by cobbles) and contains different light and flow environments. It has a slope of 1 to 2 % and was 10 to 15 m wide, 10 to 40 cm deep and 250 m in altitude. The dominant riparian trees are from

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the family Fagaceae and Taxodiaceae (higher plants with C_3 photosynthesis). Further details of this site and the DIC δ^{13}C and Δ^{14}C values have been reported in Ishikawa et al. (2012b).

We randomly collected several submerged cobbles from various habitats (e.g., open/shaded and riffle/pool), which were rinsed gently with distilled water before the periphyton was removed from the cobble surface with a brush and distilled water. The resulting slurry was placed in a 100 mL polypropylene bottle, which was frozen until further processing. As reference samples of pure aquatic and terrestrial primary producers, a filamentous green alga, *Cladophora* sp., and several fresh leaves from the Japanese blue oak, *Quercus glauca*, were collected in April. Several individuals of the mayfly larva, *Epeorus latifolium*, were collected by hand in both April and October. The larvae of *E. latifolium* have highly specialized mouths for grazing (Takemon, 2005), and their amino acid δ^{15}N values indicate that they are algal-grazing specialists (Ishikawa et al., 2014a).

2.2 Laboratory sample processing

All samples were lyophilised with a freeze drier (FDU-1200, Eyela, Tokyo, Japan) in the dark. The gut contents of *E. latifolium* larvae were removed prior to lyophilisation. The periphyton samples were ground to a fine powder with a mortar and pestle, after all large invertebrates (e.g., chironomids) had been manually removed. *Cladophora* sp. and *Q. glauca* were ground with a vibrating mill (TI-100, CMT, Fukushima, Japan). The periphyton, *Cladophora* sp. and *Q. glauca* samples were split into two vials for bulk and compound-specific isotope analyses. The vials for the bulk periphyton and *Cladophora* sp. were treated overnight with 1 M HCl solution to remove any carbonate and were then lyophilised again. The algal community in periphyton previously collected from the same site and the gut contents of *E. latifolium* were observed under a microscope.

Chlorophyll *a* was extracted using the modified method of Chikaraishi et al. (2005, 2007). Briefly, the powdered periphyton, *Cladophora* sp. and *Q. glauca* were sonicated in 100 % acetone at 0 °C for 15 min, followed by liquid–liquid (water : *n*-hexane = 3 : 1,

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v/v) extraction, with NaCl salting out to remove the lipids. The *n*-hexane layer was extracted and dried with a stream of argon, and the precipitate (i.e., pigments) was dissolved in *N*,*N*-dimethylformamide (DMF) after filtration using a syringe (0.50 mm × 25 mm ; Terumo, Tokyo, Japan) equipped with a filter (4 mm × 0.2 µm PTFE, 100 pk; Grace Dawson Discovery Science, Maryland, USA) to remove any remaining particles. The laboratory standard for chlorophyll *a* was bought commercially (lot DCL2671; Wako Pure Chemical Industries, Osaka, Japan) and the standard for phaeophytin *a* was made by adding 1 M HCl solution to the chlorophyll *a* standard.

The pigments in DMF were introduced into a high-performance liquid chromatography (HPLC) apparatus (1260 series; Agilent Technologies, California, USA), comprising a G4225A degasser, a G1312B binary pump, a G1367E autosampler, a G1316C column oven, a G1315D diode-array detector and a G1364C fraction collector. All solvents were better than HPLC-grade (Wako Pure Chemical Industries). A Zorbax XDB C18 column (5 µm/4.6 mm × 250 mm ; Agilent Technologies) and an XDB C18 guard column (5 µm/4.6 mm × 12.5 mm) were used in the first purification step. In the first step, the solvent gradient program was as follows: acetonitrile : ethyl acetate : pyridine = 75 : 25 : 0.5 ($v/v/v$) held for 5 min, then gradually changed to 50 : 50 : 0.5 ($v/v/v$) in 55 min. The flow rate of the mobile phase was 1.00 mL min^{-1}. The column oven was set at 30 °C. We identified chlorophyll *a* and phaeophytin *a* based on their retention times and UV/Vis spectral patterns, compared with those of laboratory standards (Fig. S3a and b in the Supplement).

The purified chlorophyll *a* and phaeophytin *a* were collected using the fraction collector and were dried with a stream of argon. Each fraction was dissolved in DMF and introduced into the HPLC apparatus again. A PAH column (5 µm/4.6 mm × 250 mm, Agilent Technologies) and a PAH guard column (5 µm/4.6 mm × 12.5 mm) were used in the second purification step. In the second step, the solvent gradient program was as follows: acetonitrile : ethyl acetate : pyridine = 80 : 20 : 0.5 ($v/v/v$) held for 5 min, then gradually changed to 0 : 100 : 0.5 ($v/v/v$) in 35 min. The flow rate of the mobile phase was 1.00 mL min^{-1}. The column oven was set at 15 °C. After the second step, the fractions of chlorophyll *a* and phaeophytin *a* were dried and washed with water : *n*-hexane (3 : 1, v/v). The *n*-hexane layer was carefully extracted, dried again and frozen until the isotope measurements were made. The abundances of chlorophyll *a* and phaeophytin *a* were estimated using conversion formulae between the absorbance at 660 nm and the dry weights of the laboratory standards.

2.3 δ^{13}C, δ^{15}N and Δ^{14}C measurements

The stable isotope ratios of carbon (δ^{13}C) and nitrogen (δ^{15}N) for bulk and chlorophyll *a* from periphyton, *Cladophora* sp. and *Q. glauca* samples and those for bulk *E. latifolium* samples were measured with an elemental analyser (Flash EA1112) coupled to a Delta XP isotope ratio mass spectrometer (Thermo Fisher Scientific, Massachusetts, USA) with a Conflo III interface (Thermo Fisher Scientific) modified for ultra-small-scale isotope measurements (Ogawa et al., 2010). The δ^{13}C and δ^{15}N values are reported relative to those for Vienna Pee Dee belemnite (VPDB) and atmospheric N_2 (AIR), respectively. Data were corrected using two internal standards (tyrosine: $\delta^{13}C_{VPDB} = -20.50 \pm 0.13$ ‰, $\delta^{15}N_{AIR}$: 8.44 ± 0.05 ‰; nickel octaethylporphyrin: $\delta^{13}C_{VPDB} = -34.17 \pm 0.06$ ‰; $\delta^{15}N_{AIR}$: 0.86 ± 0.03 ‰), which had been corrected against multiple international standards (Tayasu et al., 2011). The 1σ analytical precision for both δ^{13}C and δ^{15}N measurements was within 0.9 ‰.

The samples for Δ^{14}C measurements were graphitized, according to the modified methods of Kitagawa et al. (1993) and Yokoyama et al. (2010). Briefly, the bulk samples (approximately 1 mg C) and chlorophyll *a* samples (90 to 617 µg C) were combusted in an evacuated quartz tube with copper oxide at 500 °C for 30 min and at 850 °C for 2 h. The CO_2 gas was cryogenically purified in a vacuum line and reduced to graphite with hydrogen and an iron catalyst at 550 °C for 10 h. The Δ^{14}C values for the bulk samples and chlorophyll *a* samples were measured with an accelerator mass spectrometer (AMS) at Institute of Accelerator Analysis (Kanagawa, Japan; AMS lab code IAAA) and at Atmosphere and Ocean Research Institute, University of Tokyo (Chiba, Japan; AMS

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lab code YAUT), respectively. The $\Delta^{14}C$ (‰) value was defined as follows (Stuiver and Polach, 1977):

$$\Delta^{14}C(‰) = \delta^{14}C - 2(\delta^{13}C + 25)(1 + \delta^{14}C/1000) \quad (1)$$

$\Delta^{14}C$ value of the international standard (oxalic acid) took into account the radioactive decay since AD 1950 (Stuiver and Polach, 1977). The 1σ analytical precision of the $\Delta^{14}C$ measurements was within 3 ‰ for bulk and 8 ‰ for chlorophyll *a*. The HPLC procedural blank for carbon (e.g., potential contamination by column breeding), assessed with elemental analyser, was below the detection limit (< 0.177 µg C), which was lower than 0.2 % carbon in the purified chlorophyll *a* molecules used for the AMS measurement.

To determine the carbon transfer pathway in this stream ecosystem, the $\delta^{13}C$ and $\Delta^{14}C$ values for all samples were compared with those for DIC, DOC and POC collected at the same site in the Seri River in 2009 to 2010 (Ishikawa et al., 2012b, 2015).

3 Results and discussion

3.1 Sample observations

Microscopic observations show that diatoms and cyanobacteria are the dominant photoautotrophs in the periphyton community at the study site (Fig. S1 in the Supplement). Both the periphyton and gut contents of *E. latifolium* consisted not only of algal cells, but also of amorphous and unidentified particles (Fig. S2). The exuvium of small invertebrates (approximately 500 µm) was found in the periphyton matrix (Fig. S2a), the isotopic composition of which would have differed from that of pure algae. The UV/Vis spectra show different composition of photosynthetic pigments between April and October. Chlorophyll *a* (Mw, 892.5) and phaeophytin *a* (Mw, 870.6; the Mg atom is replaced by two H atoms in the centre of the tetrapyrrole ring of the chlorophyll *a* molecule) were the dominant pigments in the periphyton matrix in both April and October (Fig. S3).

Because phaeophytin *a* was more abundant than chlorophyll *a* in April, we purified phaeophytin *a* together with chlorophyll *a* and combined them for the isotope measurements. The C and N isotopic compositions of phaeophytin *a* are theoretically identical to those of chlorophyll *a* because phaeophytin *a* is an early degradation product of chlorophyll *a*, and neither a C nor an N atom is replaced in this step. The combined abundance of chlorophyll *a* and phaeophytin *a* per unit dry weight was greater in October than in April, indicating that the algal biomass of the periphyton community was greater in October than in April (Table S2).

3.2 ^{13}C composition

The periphyton $\delta^{13}C_{bulk}$ and $\delta^{13}C_{chl}$ values were −20.7 and −20.0 ‰, respectively, in April, and −26.2 and −26.0 ‰, respectively, in October (Fig. 1). The algal-grazer *E. latifolium* $\delta^{13}C_{bulk}$ values were −26.6 and −26.5 ‰ in April and October (Fig. 1), respectively. In October, the periphyton $\delta^{13}C_{bulk}$ and $\delta^{13}C_{chl}$ values were close to the *E. latifolium* $\delta^{13}C_{bulk}$ value. In contrast, neither the periphyton $\delta^{13}C_{bulk}$ nor $\delta^{13}C_{chl}$ value was close to the *E. latifolium* $\delta^{13}C_{bulk}$ value in April, indicating that the $\delta^{13}C$ value does not faithfully trace the carbon transfer between the primary producers and the primary consumers. This is partly because the periphyton $\delta^{13}C_{bulk}$ values vary from −32 to −16 ‰ among stream habitats (e.g., open/shaded and riffle/pool) in this study site, due to the variable isotopic fractionation between DIC and algae (Ishikawa et al., 2012b). Such a large variation in periphyton $\delta^{13}C_{bulk}$ values on a small spatial scale may cause an inconsistency in $\delta^{13}C$ between periphyton (primary producers) and *E. latifolium* (primary consumers).

A mismatch between the $\delta^{13}C_{bulk}$ values for periphyton and grazers is often observed (Dekar et al., 2009), although ^{13}C is not enriched through the trophic levels (Vander Zanden and Rasmussen, 2001). There are four independent scenarios that explain our $\delta^{13}C$ results. Firstly, *E. latifolium* assimilates the ^{13}C-depleted fraction in periphyton. Secondly, *E. latifolium* assimilates the terrestrial organic matter, which is more ^{13}C-depleted than the periphyton. Thirdly, the periphyton $\delta^{13}C_{bulk}$ and $\delta^{13}C_{chl}$ values varied by 6 ‰, whereas the *E. latifolium* $\delta^{13}C_{bulk}$ values did not change greatly between April and October, suggesting that primary consumers integrate temporal fluctuations in $\delta^{13}C$ values for primary producers. Finally, the $\delta^{13}C_{chl}$ value is not a reliable proxy for bulk algae because the $\delta^{13}C_{chl}$ value is affected by the isotopic fractionation that occurs during chlorophyll *a* biosynthesis. To provide a more precise estimate of algal carbon, the $\Delta^{14}C_{chl}$ signature is useful because it is corrected for isotopic fractionation by $\delta^{13}C$ in Eq. (1) (Stuiver and Polach, 1977).

$\delta^{13}C_{bulk}$ and $\delta^{13}C_{chl}$ values were −23.0 and −24.7 ‰, respectively, for *Cladophora* sp. and −30.9 and −32.0 ‰, respectively, for *Q. glauca* (Fig. 1). The $\delta^{13}C_{chl}$ value for primary producers is controlled by the $\delta^{13}C$ value for their carbon source (i.e., DIC for *Cladophora* sp. and atmospheric CO_2 for *Q. glauca*) and by internal isotopic fractionation between bulk cells and chlorophyll *a* molecules. Sachs et al. (1999) reported that $\delta^{13}C_{chl}$ values for a cultivated green alga *Dunaliella tertiolecta* were 0.5 to 4.0 ‰ lower than those for their bulk cells, which is consistent with our *Cladophora* sp. data. Chikaraishi et al. (2005) reported the same $\delta^{13}C_{bulk}$ value (−30.9 ‰) for the fresh leaves of the Mongolian oak *Q. mongolica* as for our *Q. glauca* data. In contrast, in this study, the *Q. glauca* $\delta^{13}C_{chl}$ value (−32.0 ‰) was lower than that for *Q. mongolica* (−29.2 ‰) reported in Chikaraishi et al. (2005).

3.3 ^{15}N composition

The periphyton $\delta^{15}N_{bulk}$ and $\delta^{15}N_{chl}$ values were −5.7 and −1.5 ‰, respectively, in April, and −1.7 and +0.5 ‰, respectively, in October (Fig. 1). The algal-grazer *E. latifolium* $\delta^{15}N_{bulk}$ values (−3.9 ‰ in April and +1.4 ‰ in October) were 1.8 to 2.9 ‰ higher than the periphyton $\delta^{15}N_{bulk}$ values. The $\delta^{15}N_{bulk}$ and $\delta^{15}N_{chl}$ values were −4.3 and −6.0 ‰, respectively, for *Cladophora* sp. and −0.8 and −0.2 ‰, respectively, for *Q. glauca* (Fig. 1). Sachs et al. (1999) reported that the $\delta^{15}N_{chl}$ values were 2 to 9 ‰ lower than the $\delta^{15}N_{bulk}$ values for phytoplankton because of the isotopic fractionation

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that occurs during chlorophyll *a* biosynthesis. Kennicutt et al. (1992), on the other hand, reported that the $\delta^{15}N_{chl}$ values were relatively close to the $\delta^{15}N_{bulk}$ values for terrestrial C_3 plants. Therefore, the relationships between $\delta^{15}N_{bulk}$ and $\delta^{15}N_{chl}$ values for *Cladophora* sp. and *Q. glauca* are consistent with those for previous studies. In contrast, the periphyton $\delta^{15}N_{chl}$ values were 2.2 to 4.2 ‰ higher than their $\delta^{15}N_{bulk}$ values. This result might be attributable to the presence of cyanobacteria (e.g., *Oscillatoria* sp. or *Homoeothrix* sp., Fig. S1) in the periphyton community, because the $\delta^{15}N_{bulk}$ and $\delta^{15}N_{chl}$ values for cyanobacteria are usually different from those for algae (Beaumont et al., 2000).

3.4 ^{14}C composition

The $\delta^{13}C$ and $\Delta^{14}C$ values for DIC at the same study site in the Seri River have been reported previously as −7.2 ± 0.2 and −217 ± 30.7 ‰, respectively (four-season mean ± SD, N = 16; Ishikawa et al., 2012b; Figs. 1 and 2). These values are balanced by the mixing of weathered carbonates ($\delta^{13}C$ = +3.9 ± 0.3 ‰ and $\Delta^{14}C$ = −1000 ‰), dissolved atmospheric CO_2 ($\delta^{13}C$ and $\Delta^{14}C$ are approximately −8 ‰ and +30 ‰, respectively, in 2013) and mineralized organic materials (DOC: $\delta^{13}C$ = −24.2 ± 2.9 ‰, $\Delta^{14}C$ = −248 ± 110 ‰; POC: $\delta^{13}C$ = −25.0 ± 3.4 ‰, $\Delta^{14}C$ = −109 ± 52 ‰) (four-season mean ±SD, N = 4 for each fraction) at the study site (Ishikawa et al., 2015; Figs. 1 and 2).

The periphyton $\Delta^{14}C_{bulk}$ and $\Delta^{14}C_{chl}$ values (mean of the repeated measurements ±1σ analytical precision) were −228 ± 2.3 and −258 ± 4.8 ‰, respectively, in April, and −179 ± 2.2 and −190 ± 6.1 ‰, respectively, in October, showing that chlorophyll *a* is slightly depleted in ^{14}C relative to the bulk of the periphyton (Fig. 1). In particular, the periphyton $\Delta^{14}C_{chl}$ value in April was lower than the seasonal range of DIC $\Delta^{14}C$ (Fig. 1). There are two possible explanations of the periphyton $\Delta^{14}C_{chl}$ value in April. Firstly, periphytic algae assimilate CO_2 dissolved from the bedrock limestone at the biofilm–bedrock boundary, in addition to water column DIC. Because respira-

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tory CO_2 and organic acids can mediate carbonate weathering (Berner et al., 1983), ^{14}C-dead (i.e., $\Delta^{14}C = -1000$ ‰) CO_2 derived from carbonates may enter the algae. Secondly, periphytic algae collect phytol from ^{14}C-depleted DOC or POC and use it for the biosynthesis of the chlorophyll *a* molecule, which results in a low $\Delta^{14}C_{chl}$ value. In fact, the cyanobacterium *Synechocystis* sp. have a phytol-recycling pathway during chlorophyll *a* biosynthesis (Vavilin and Vermaas, 2007).

The $\Delta^{14}C_{bulk}$ and $\Delta^{14}C_{chl}$ values were -199 ± 2.7 and -210 ± 6.8 ‰, respectively, for *Cladophora* sp. and $+27 \pm 2.3$ and -10 ± 7.3 ‰, respectively, for *Q. glauca* (Fig. 1). The $\Delta^{14}C_{bulk}$ and $\Delta^{14}C_{chl}$ values for the pure primary producers were not expected to differ greatly, but the *Q. glauca* $\Delta^{14}C_{chl}$ value was 37 ‰ lower than its $\Delta^{14}C_{bulk}$ value (Fig. 1). This result suggests that *Q. glauca* synthesizes chlorophyll *a* using not only atmospheric CO_2, but also aged (^{14}C-depleted) soil CO_2 and/or organic carbon taken up through its roots, as reported in various terrestrial plants (Brüggemann et al., 2011; Bloemen et al., 2013). An alternative hypothesis is that *Q. glauca* and probably other terrestrial plants recycle the chlorophyll *a* molecule through the salvage pathway of phytol metabolism, as reported in *Arabidopsis* seedlings (Ischebeck et al., 2006).

Because the periphyton $\Delta^{14}C_{chl}$ value and the *Q. glauca* $\Delta^{14}C_{bulk}$ value represent the aquatic and terrestrial end members, respectively, a two-source mixing model can estimate relative abundances of aquatic (e.g., algae) and terrestrial (e.g., leaf detritus) carbon fractions in periphyton. The results show that the periphyton bulk matrix consisted of 89 % (April) to 95 % (October) aquatic carbon and 5 % (October) to 11 % (April) terrestrial carbon. The *E. latifolium* $\Delta^{14}C_{bulk}$ values (-215 ± 2.3 ‰ in April and -199 ± 2.2 ‰ in October) were within the range of periphyton $\Delta^{14}C$ values (Fig. 1). The April *E. latifolium* $\Delta^{14}C_{bulk}$ value was closer to the periphyton $\Delta^{14}C_{bulk}$ value than to its $\Delta^{14}C_{chl}$ value, suggesting that *E. latifolium* assimilates not only ^{14}C-depleted aquatic sources, but also ^{14}C-enriched terrestrial sources in April. In contrast, the October *E. latifolium* $\Delta^{14}C_{bulk}$ value was closer to the periphyton $\Delta^{14}C_{chl}$ value than to its $\Delta^{14}C_{bulk}$ value, suggesting that *E. latifolium* primarily assimilates aquatic sources in October. This seasonal variation may be attributed to the higher chlorophyll *a* abundance per unit dry weight in October, and/or to the higher terrestrial flux associated with the input of snow melt in April.

3.5 Implications of this study

The periphyton $\delta^{13}C_{chl}$ and $\Delta^{14}C_{chl}$ values are generally consistent with its $\delta^{13}C_{bulk}$ and $\Delta^{14}C_{bulk}$ values (Figs. 1 and 2) in this study. Although this has been assumed in previous studies, without direct evidence, our results indicate that the periphyton $\delta^{13}C_{bulk}$ and $\Delta^{14}C_{bulk}$ values can be used as a surrogate for the photosynthetic algal community in periphyton (Fig. 3). However, there remain some uncertainties in our data, such as the results that the $\delta^{15}N_{chl}$ values were higher than $\delta^{15}N_{bulk}$ values in periphyton and that the $\Delta^{14}C_{chl}$ values were slightly lower than the $\Delta^{14}C_{bulk}$ values. Furthermore, because the Seri River is relatively productive, diatoms and cyanobacteria dominate the periphyton community rather than non-algal materials (e.g., leaf detritus) (Fig. S1). Therefore, future studies should attempt to generalise our results and test how much periphyton $\delta^{13}C$, $\delta^{15}N$ and $\Delta^{14}C$ values differ between bulk and chlorophyll *a* in multiple streams and rivers. In particular, data collected from less productive streams, where the terrestrial detritus is more abundant than the algae/cyanobacteria in the periphyton, should be examined to confirm the advantage of using the isotopic composition of chlorophyll *a* in this type of analysis.

Compound-specific stable isotope and radiocarbon analyses are promising tools for the precise estimation of the sources, dynamics and turnover of various organic molecules (Hayes et al., 1987; Eglinton et al., 1996; Jochmann and Schmidt, 2012; Ohkouchi et al., 2015). The isotopic composition of chlorophyll *a* can be used not only in stream ecosystems, but also in coastal ecosystems, where benthic biofilms (i.e., mixtures of algae and other heterotrophs) are important food sources for invertebrates, fish and birds (Kuwae et al., 2008, 2012). Chlorophyll *a* is a unique biomarker of in situ photoautotrophs and more accurate than other biochemical compounds (e.g., lipids and amino acids) because it is immediately degraded in the inactive state (Carpenter

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et al., 1986; Amir-Shapira et al., 1987; Matile et al., 1996). We conclude that the chlorophyll *a* specific $\Delta^{14}C$, $\delta^{13}C$ and $\delta^{15}N$ values are useful tracers for precisely estimating the sources of carbon and nitrogen in complex ecosystems, in which heterogeneous resources (e.g., aquatic and terrestrial organic matters) are mixed.

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Author contributions. N. F. Ishikawa conceived the study design and conducted fieldwork. N. F. Ishikawa and H. Suga conducted pigment purification using HPLC. N. O. Ogawa conducted $\delta^{13}C$ and $\delta^{15}N$ analyses using EA/IRMS. M. Yamane and Y. Yokoyama conducted $\Delta^{14}C$ analysis using AMS. All authors participated discussion. N. F. Ishikawa and N. Ohkouchi wrote the manuscript.

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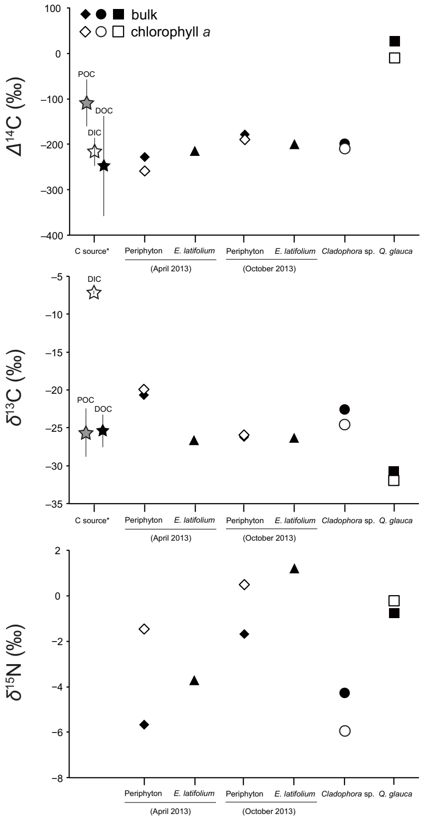

Figure 1. The $\Delta^{14}C_{bulk}$, $\delta^{13}C_{bulk}$ and $\delta^{15}N_{bulk}$ values (shaded symbols) for periphyton (diamonds), *Cladophora* sp. (aquatic primary producer; circle), *Q. glauca* (terrestrial primary producer; square) and *E. latifolium* (algal grazer; triangles) and the $\Delta^{14}C_{chl}$, $\delta^{13}C_{chl}$ and $\delta^{15}N_{chl}$ values (open symbols) for periphyton, *Cladophora* sp. and *Q. glauca*. DIC: dissolved inorganic carbon; DOC: dissolved organic carbon; POC: particulate organic carbon. *Data from Ishikawa et al. (2012b, 2015).

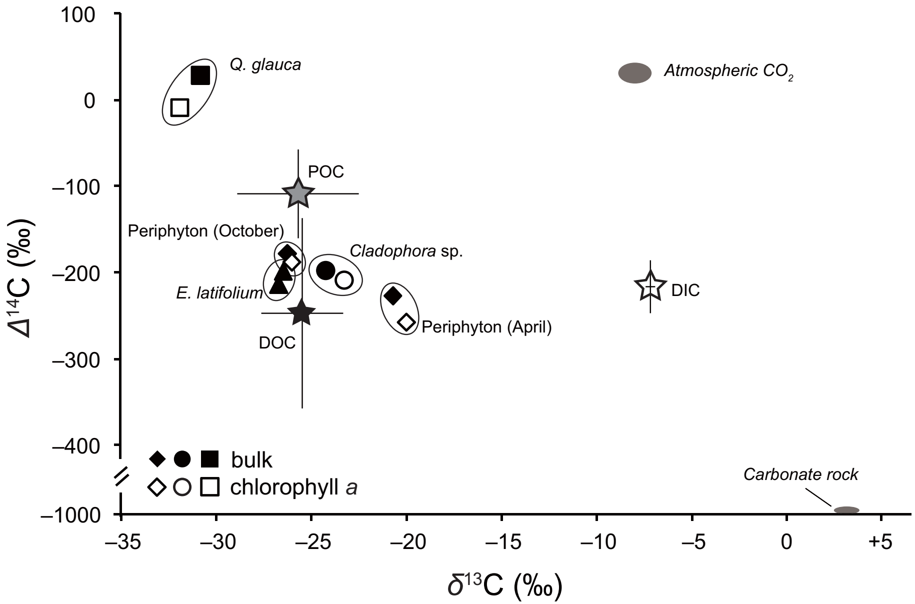

Figure 2. Biplot of δ^{13}C and Δ^{14}C data. Carbonate rocks in the Seri River ($\delta^{13}C = +3.9 \pm 0.3$ ‰ and $\Delta^{14}C = -1000$ ‰) (Ishikawa et al., 2015) and atmospheric CO_2 (δ^{13}C and Δ^{14}C are approximately −8 and +30 ‰, respectively, in 2013) are also shown as end members.

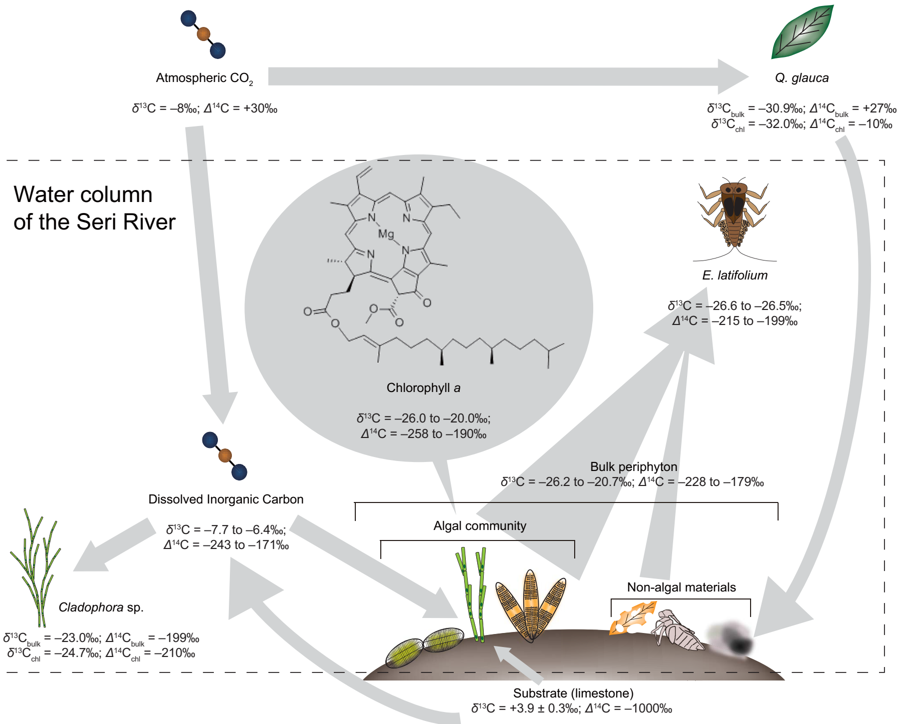

Figure 3. Schematic view of the carbon cycle at the study site (Seri River) constrained by $\delta^{13}C$ and $\Delta^{14}C$.

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