Dear the editor and referees,

We are grateful to the constructive comments from three anonymous referees on our paper. We also thank the associate editor Dr. Tom J. Battin for handling the manuscript. Below we responded to each of the referees' comments and described how we revised the manuscript. The numbers of page and line (e.g., P10L23) in our response are for the revised manuscript (the revised sentences are highlighted). We believe that the revised manuscript has been greatly improved in accordance with the referees' valuable suggestions. In case we disagree with a specific recommendation, further explanations supporting our approach were made.

Sincerely,

Naoto F. Ishikawa

Responses to the comments from Referee #1

(RC: Referee comment; AC: Author comment)

(RC) The manuscript written by Ishikawa et al. reported chlorophyll a specific Δ14C, δ13C and δ15N values in stream periphyton. The information is new and provides valuable insights on the study of stream food web. I have only some minor comments and questions to the authors.

(AC) Thank you for your valuable comments. Please see our responses to your comments below.

(RC) P.11096 l. 17 1-sigma of the measurement was 0.9 permil, which seems high especially for bulk analysis. I consider the "ultra-small-scale" analysis is required for chlorophyll a, but the authors can provide more precise data for other samples.

(AC) We revised the sentence as "The 1σ analytical precision for both δ^{13}C and δ^{15}N measurements was within 0.2‰ for bulk and with 0.9‰ for chlorophyll *a*.". Please see P7L1-3.

(RC) P.11101 ll.10-16 The authors suggested two possible mechanisms explaining the difference in Δ14C values between bulk and chlorophyll in terrestrial plants. However, both explanations are difficult to understand why chlorophyll has such an "old" signal, compared to the fact that Δ14C value of bulk tissue is almost identical to that of ambient CO2. Especially, the latter mechanism is difficult understand. The Δ14C value of chlorophyll will be higher than that of bulk tissue if the salvage pathway occurs.

(AC) We revised this paragraph explaining the differences in Δ^{14}C between bulk and chlorophyll *a* in *Q. glauca*. To support our explanation, two references (Trumbore and Zheng 1996; Koarashi et al., 2009) showing that soil organic carbon does not necessarily have modern carbon were added. Furthermore, we discussed that carbon in chlorophyll *a* molecule may be originated from various sources because its biosynthesis has multiple channels to acquire carbon. Please see P10L23-P11L8.

(RC) Section 3.5 Implications of this study: the authors concluded that the δ13C and Δ14C values of bulk periphyton can be used as a surrogate of those of photosynthetic algal community in periphyton, which seems a good news to many ecologists who are difficult to access the technique. However, the authors need to stress on potential advantages of the technique in the study of stream ecosystems, where the study was conducted. The final paragraph is rather easy to understand, but the manuscript focused on stream food web. I don't think a potential application to "less productive stream" (p.11102 l.15) is an attractive example. Need more explanations.

(AC) We revised section 3.5 to stress on potential advantages of chlorophyll specific isotope analysis for not only stream ecology, but also biogeochemical science. A brief note on pitfalls in the methodology was also added. Please see section 3.5.

End of responses to the comments from Referee #1

Responses to the comments from Referee #2

(RC: Referee comment; AC: Author comment)

(RC) The manuscript by Ishikawa et al. showed that chlorophyll a compound-specific Δ14C, δ13C and δ15N values in stream periphyton. The data and implications are novel and may be useful for future stream food-web studies. The manuscript was well written and the results are clear, but I have a few concerns on the manuscript.

(AC) Thank you for your valuable comments. Please see our responses to your comments below.

(RC) 1) P11065L21 It is unclear why you used the both chlorophyll a and phaeophytin a. If you used the both you should explain the reasons.

(AC) We moved the sentence explaining why both chlorophyll *a* and phaeophytin *a* were used from section 3.1 to section 2.2. Please see P6L5-9.

(RC) 2) The mechanisms to explain the differences in Δ14C between bulk and chlorophyll-a specific in litters were unclear. I am interested in the data because I guessed the Δ14C of bulk would take lower values than that of chl-a. So, I recommend you to discuss more about the phenomenon.

(AC) We revised this paragraph explaining the differences in Δ^{14}C between bulk and chlorophyll *a* in *Q. glauca*. To support our explanation, two references (Trumbore and Zheng 1996; Koarashi et al., 2009) showing that soil organic carbon does not necessarily have modern carbon were added. Furthermore, we discussed that carbon in chlorophyll *a* molecule may be originated from various sources because its biosynthesis has multiple channels to acquire carbon. Please see P10L23-P11L8.

(RC) 3) I understood some of implications of this study in the last paragraph. But in the most of the periphyton samples, the isotope values of the bulk and chl-a specific are very close. I think from this study, we should not consider the chl-a specific isotopes in

the most cases. You should emphasize which situation the chl-a specific isotopes are useful to analyze stream food web, e.g., habitats and algal compositions.

(AC) We revised section 3.5 to stress on potential advantages of chlorophyll specific isotope analysis for not only stream ecology, but also aquatic biogeochemical science. A brief note on pitfalls in the methodology was also added. Please see section 3.5.

End of responses to the comments from Referee #2

Responses to the comments from Referee #3

(RC: Referee comment; AC: Author comment)

(RC) This study investigated the chlorophyll-a specific isotopic compositions in stream periphyton to examine whether the bulk isotopic compositions of periphyton could be used as representative of aquatic producers. The results showed that periphyton chlorophylla exhibited 13C and 14C values similar to the bulk tissue, but had higher 15N value than the bulk sample. The difference in 15N value between chlorophyll-a and bulk sample was attributed to N isotopic fractionation during chlorophyll-a biosynthesis and incorporation of cyanobacteria tissue into periphyton. Because of the novelty of measurement on chlorophyll-a specific isotopic compositions of 13C, 14C and 15N of stream periphyton, I would like to recommend this manuscript for Biogeosciences. However, I think that there are some issues to be addressed before final publication.

(AC) Thank you for your valuable comments. Please see our responses to your comments below.

(RC) For example, the authors calculated the relative contribution of algal carbon and terrestrial organic carbon to periphyton based on 14C values of bulk periphyton, chlorophyll a, and terrestrial plant for each season (April and October). They concluded that the periphyton consisted of 89 – 95 % algal carbon. I wonder if this is a meaningful and reliable calculation. The algal portion of periphyton should consist of both alive and dead (aged) algal tissues. Further, 14C value in periphyton chlorophyll-a changed largely (ca. 60 permil) differed between April and October. Therefore, I suppose that the difference in 14C values of bulk periphyton and chlorophyll-a could be accounted for not only by terrestrial organic carbon incorporation but also by the seasonal variation in 14C of chlorophyll-a. Actually, Cladophora sp., the aquatic primary producer, also presented a difference (ca. 10 permil) in 14C between bulk periphyton and chlorophyll-a. The difference is comparable to that in periphyton in October. I think that it would be necessary to consider more carefully about the premise of the calculation.

(AC) Thank you for this comment. Assuming that our April and October data represent seasonal variation, bulk periphyton $\Delta^{14}C$ values in April and October can be explained by both seasonal variation in aquatic end member (as indicated by chlorophyll *a* $\Delta^{14}C$ in periphyton) and relative contributions of the aquatic and terrestrial end members to periphyton bulk matrix. As you pointed out, chlorophyll *a* $\Delta^{14}C$ in periphyton in April was largely different from that in October. However, our long-term monitoring indicates that frequent flooding renews benthic environment and causes rapid turnover of algal community in periphyton in this stream. Textbooks in this field (e.g., Allan and Castillo 2007 Stream Ecology) state that turnover of periphytic algae is generally 3-6 weeks. As far as we know, chlorophyll *a* in April periphyton should not be remained in October periphyton and chlorophyll *a* $\Delta^{14}C$ value for living algae should not be greatly different from that for dead algae. We agree with your comment that the *Cladophora* sp. $\Delta^{14}C$ difference between bulk and chlorophyll *a* (10‰) is comparable to that in periphyton in October (10‰). However, this result does not indicate that October periphyton is consisted of 100% aquatic carbon because bulk and chlorophyll *a* $\Delta^{14}C$ values for October periphyton are different from those for *Cladophora* sp. As $\Delta^{14}C$ value of terrestrial end member (*Q. glauca*) is fixed in this study, a separate two-source mixing model should be applied to each of April and October. Based on your comments, two assumptions in our model were added to text to validate our approach and we revised several sentences. Please see P11L9-26.

(RC) Additionally, 14C value of chlorophyll-a of terrestrial plant leaves (-10 permil) was much lower than that of bulk 14C (27 permil). The difference was considered to be because of use of old soil CO2 and soil organic carbon. It should be extremely interesting if the plant can have access to such an old carbon source. The two cited papers (Bloemen et al. and Bruggemann et al.) indeed described the potential importance of these carbon sources for plant production, but these two references did not demonstrate that plants could use such an old carbon for primary production. To my knowledge, most of previous 14C studies have shown that respired soil CO2 and dissolved soil organic carbon have modern carbon. The recycle of phytol was also used to explain the 14C difference between chlorophyll-a and bulk plant leaves. I like this idea but it is difficult to believe that plant reuse such an old phytol to synthesize chlorophyll-a. Please consider presenting more convincing evidence to support the

authors' idea.

(AC) We revised this paragraph explaining the differences in $\Delta^{14}C$ between bulk and chlorophyll *a* in *Q. glauca*. To support our explanation, two references (Trumbore and Zheng 1996; Koarashi et al., 2009) showing that soil organic carbon does not necessarily have modern carbon were added. Furthermore, we discussed that carbon in chlorophyll *a* molecule may be originated from various sources because its biosynthesis has multiple channels to acquire carbon. Please see P10L23-P11L8.

Minor comments

(RC) P11090: Please consider describing the rationale of this study in the first sentence of Abstract.

(AC) We revised the first two sentences in Abstract as "Periphytic algae attached to a streambed substrate (periphyton) are an important primary producer in stream ecosystems. We determined the isotopic composition of chlorophyll *a* in periphyton collected from a stream flowing on limestone bedrock in the Seri River, central Japan.". Please see P1L13-15.

(RC) P11090L10, P11098L15: The authors stated that 13C of periphyton do not trace carbon transfer between primary producers and primary consumers. However, the 13C data clearly indicated that the mayfly larva did not subsist on C of periphyton that was investigated. Please clarify what kind of C flow the authors intended to mention.

(AC) We deleted this statement in both Abstract and section 3.2. This deletion did not influence our conclusion. Please see P1L20 and P8L16.

(RC) P11090L15: mixture of only two sources (carbonates and atmospheric CO2)? What about CO2 derived from aquatic and terrestrial organic matter?

(AC) We added ", CO_2 derived from aquatic and terrestrial organic matters (variable $\Delta^{14}C$)" after "weathered carbonates ($\Delta^{14}C = -1000‰$)". Please see P1L24.

(RC) P11091L26: Peripyton 14C is “often” derived

(AC) We added “often” after “Periphyton Δ^{14}C is”. Please see P2L28.

(RC) P11094L23: washed with H2O after HCl treatment?

(AC) We added “washed and” after “carbonate and were”. Please see P5L9.

(RC) P11094L24: when was the periphyton sample collected?

(AC) We added “(November 2008)” after “the same site”. Please see P5L10.

(RC) P11095L8: Please describe briefly how to confirm that the product was phaeophytin-a.

(AC) We added “Absorption spectra of our laboratory standards were consistent with those reported in literatures (Chikaraishi et al., 2007; Tyler et al., 2010).” after “chlorophyll *a* standard.”. Please see P5L22-23.

(RC) P11096: Please add more explanations about how to transfer the dried chlorophyll-a samples to tin capsules for 13C and 15N and quartz tubes for 14C measurement.

(AC) We added “The dried chlorophyll *a* and phaeophytin *a* were dissolved in dichloromethane and transferred to tin capsules for δ^{13}C and δ^{15}N measurements or to quartz tubes for Δ^{14}C measurements. The tin capsules and quartz tubes were dried again prior to measurements.” as the last sentence of section 2.2. Please see P6L20-22.

(RC) P11099L5: proxy for “13C” of bulk algae.

(AC) We added “δ^{13}C of” after “reliable proxy for”. Please see P8L30.

(RC) P11101L3: It is a great idea. But are there any studies demonstrating that an algae can collect phytol from DOC or POC?

(AC) We deleted phytol recycling mechanisms due to the lack of convincing evidence and revised the sentences as "Secondly, heterotrophs such as fungi and bacteria in periphyton community consume ambient DOC and release CO_2 during their respiration (Fischer 2003). The CO_2 derived from heterotrophic respiration of DOC may be another ^{14}C-depleted carbon source that is utilized by periphytic algae for photosynthesis.". Please see P10L19-22.

(RC) Fig.1 and 2.: Please indicate what the error bars stand for.

(AC) We added "Error bars indicate standard deviation (N = 4)." in legends of Figures. 1 and 2. Please see P22L7 and P23L6.

End of responses to the comments from Referee #3