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Biogeosciences
Discussions

Interactive comment on "Carbon isotope fractionation in developing natural phototrophic biofilms" *by* M. Staal et al.

Anonymous Referee #1

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General comments

The manuscript adresses the issue of carbon isotope fractionation in laboratory-grown phototrophic biofilms. The biofilm systems studied were grown from natural inoculums but are still appealingly simple fully phototrophic systems that allow testing of hypotheses in a reductionist manner. The authors propose two biofilm-internal processes: (a) mass transfer limitations due to increasing biofilm thickness and increasing C-demand, and (b) heterotrophic activity, to control carbon isotope fractionation and shape bulk biofilm δ^{13}C signatures. This hypothesis is quite different from former concepts of C-fractionation in biofilms which were mainly adressing external controls such as external carbon sources or diffusion limitation through boundary layers of varying thickness. In this sense the paper is a valuable contribution to our understanding of C-fractionation

in biofilms, but also increases our knowledge about the mechanics of biofilm-internal processes.

The paper, however, suffers from a couple of deficits:

First, the experimental design seems poorly replicated while at the same time trying to cover too many treatment combinations defined by 4 (!) factors: irradiance, flow rate, growth phase and marine/freshwater. The low level of replication for each treatment combination (essentially only 1 measurement) prevents successful testing of the various possible controlling factors. It necessarily leads to pooling of data from various treatments which then shows relatively high variability which cannot be separated into randomly induced variability and treatment-induced variability. The observed trends thus show tremendous scatter suggesting a high level of uncertainty for the conclusions. I cannot help to note that the statistical analyses as well as the report is relatively poor: information about replication is often missing, important statistics are not reported, statistics behind error bars cannot be identified, it is hard to follow which data was pooled for a certain analyses, the application of certain methods such as ANOVA cannot be supported. Eventually a smarter statistical analyses could greatly improve the paper, but it will suffer from poor replication.

Second, the data seems to support the conclusions in a rather vague manner only. Some of the trends appear unsure, display very high scatter, and alternative trends suggest themselves. Surely the low level of replication takes its toll. The two main alternative processes which could help to (additionally) explain the observed patterns, namely growth rate and pH of the medium, are poorly dealt with. Reliability of the growth rate estimates can be doubted and virtually no data is shown for the growth rates, even though the growth rate argument plays a major role. The logic behind the proposed way to deal with the medium's pH changes by sampling at the end of the run cannot be followed.

Third, the paper seems to have been written rather hastily. A long list of technical cor-

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rections is accompanied by some serious deficits in the graphical displays. Readability, interpretation and cross-comparison of figures could be much improved with only little extra-effort.

Generally I have the feeling that some valid and valuable conclusions from the work are suggested but could be reported in a more concise and clearer manner. In fact, the "main message", namely C-fractionation in biofilms as a balanced result of two biofilm-internal processes: C-demand and thickness-controlled mass transfer, and the amount of heterotrophic activity, proposes itself clearly but the paper lacks its concise citation.

Specific comments

Reviewing fractionation (page 71, lines 20-22): It is quite unclear if the permil-values given are actual biomass δ^{13}C values or fractionation values. Also I expect the range given (-29 to -21 permil) to be larger if these are biomass δ^{13}C values. In fact, the expected minimum δ^{13}C under non-limiting conditions is -37 permil in an aquatic environment (Hecky and Hesslein 1995) and values of δ^{13}C for aquatic primary producers outside of the given range are reported in the literature (e.g., Hecky and Hesslein 1995, Finlay et al. 1999).

Incubator design and experimental setup (page 73, lines 20ff): A couple of points are slightly unclear: One incubator has one lane ore more? Are there any replications at the level of the incubator? Medium was mixed between the incubators with different treatments of flow rate and/or irradiance? Also it would be really worth to report approximate flow velocites corresponding to the two flow rates, as velocity and associated parameters (e.g., boundary layer thickness) were reported to be significant factors for biomass δ^{13}C of biofilm in previous studies.

Light attenuation as a proxy for biomass: How well did the dry weight correlate with light absorption? (page 74, line 4-5). How old were the biofilms when sampled at 50% and 90% light absorption? (page 74, lines 7ff) Or were they sampled at different times and light absorption was the only criterion? When a linear relationship between light

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absorption and wet weight was only found up to absorption values of 80-85%, then how can actual growth rates (not maximum growth rates) be reliably estimated for biofilms with absorption values >85% (of which there are quite a lot as can be seen from the figures 2 and 3)? And how can one be sure that the biofilm is actually in mature state with no net-growth anymore?

Growth rates (page 77, lines 13ff; page 80, lines 25ff): It is quite unreasonable to compare growth rates of freshwater and marine biofilms since incubation temperatures were different. Also, are the "minute" differences significant? If not, then it is probably better to state that no differences could be found. A short sentence about the quality of growth rate estimation (maybe only minimum r^2 and/or range of r^2-values of logistic growth models) should be included here. In fact, since growth rates play an important role in the following arguments, a graphical display of growth rate estimations, a graph showing one or more representative example(s) of incubation time versus light attenuation (as biomass proxy) or a plot of time versus growth rate estimates is what the reader looks for. It would also help if the reader could identify the times of sampling (3 growth phases) on a growth curve. Since growth rates are a function of biomass (or light attenuation), it is hard to believe that no meaningful relationships between actual growth rates and δ^{13}C values could be found. Because this is a central point in the paper, graphical displays demonstrating the lack of correlation would be helpful (or at least results of non-significant correlation analyses). In the discussion (page 81, lines 10ff) the authors report δ^{13}C to be lowest during the initial growth phase in freshwater biofilms. In fact, a couple of biofilm samples with light absorption values close to 50%, presumably sampled during the exponential phase, have lower δ^{13}C-values and some graphs of Fig. 2 suggest a curvilinear rather than a monotonically increasing trend. And when the reader is ready to accept the suggested trend and follows the argument presented in lines 14-18 of page 81, one really strongly wonders why no relationship of δ^{13}C with growth rate was found. Generally for phototrophic biofilms (with light as the only ultimate energy source), one would assume photosynthesis rates to correlate with growth rates, especially during development of the biofilms and under conditions

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of low heterotrophic activity as in the freshwater biofilms. Maybe there is a growth rate estimation problem (see comment on light attenuation as proxy for biomass)?

Statistical considerations: Crucial information about how often, when and with how many replicates biofilm was sampled in the various treatments is missing (page 74, lines 14ff). The same information should be given for DIC-samples. The statistical analyses used cannot be followed easily and often sample size is unclear. What is the sample size (or d.f.) for the one-way ANOVA run on page 78, line 10? Why was an ANOVA used since in this case only two groups are compared? How was the possible effect of irradiance tested (page 78, line11)? Another one-way ANOVA with the same data pooled in a different way? Again, what was the sample size? Why not a two-way ANOVA with marine/freshwater and irradiance levels? Also, I have a hard time recognizing the trends mentioned on page 78, lines 11ff in Fig. 4: Again, the sample size is unclear. Is it only 2 for each growth phase and irradiance combination? How were error bars in Fig. 4 calculated? Why are specific treatments (growth phase and irradiance combinations) missing from both plots? I have to note that none of the hypothesized trends was tested or the results of statistical analyses are missing. I have to strongly discourage from the use of a one-way ANOVA to test for differences between two irradiance treatments in the mature stage of the marine runs (page 78, lines 19-20). A sample size of 3 is really small, ANOVA-conditions cannot be checked under these circumstances and the fact that “replicates” seem to stem from various pooled treatments does not help, either! Whatever type of analysis is used, the usual statistics should be reported.

δ^{13}C of DIC (page 78): Was the δ^{13}C of DIC measured only in the beginning of the experiment? Recognizing the pronounced changes of the medium’s pH during incubation and the knowledge of strong δ^{13}C differences (8 permil) between $CO_{2(aq)}$ and HCO_3, one wonders if δ^{13}C of DIC may change over time.

Sampling biofilm at end of run (page 78-79): Why would the biofilm samples taken at the end of the run be “low biomass samples”? At the time of sampling the old-

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est of these samples (replacement slides for sampling of initial growth phase) must be already older than 20 days. Also the graphs (Fig. 2e and 3e) suggest plenty of samples with light absorption values close to 100%, thus having relatively high biofilm biomass. When these are still considered low biomass samples, I strongly suspect light absorption to be a bad proxy for biomass! Also, I cannot follow the logic why these samples could help to circumvent the effect of the medium's pH changes during growth. Even though no information about the time of sampling for the exponential and mature growth phases is provided, these sampling times will certainly be well after 10 days, the time of sampling for the initial growth phase. During the time period from day 10 to approximately day 33 (the supposed end of the experiment as extracted from Fig. 5) the incubators demonstrate a high variability of pH over time but also between irradiance treatments. For the graphs in Fig. 2 and Fig. 3, which hold the central graphical arguments for the paper, data from the various irradiance treatments was pooled, sometimes even though distinct clusters (especially pronounced in Fig. 2e and Fig. 3e) can be recognized. With the information given it is virtually impossible to exclude an effect of the medium's pH! In fact, one wonders how a graph of pH of medium (maybe averaged over the growth period of the biofilm) versus biofilm δ^{13}C would look like!

Effect of flow on δ^{13}C and heterotrophic activity (page 82, lines 18ff): The authors attribute the lack of a relationship between flow rate and δ^{13}C of biofilm, as found in this study, to the fact that compared to other studies a completely different flow regime may have been used. Sadly, flow velocities in the incubators or related parameters such as thickness of the diffusive boundary layer are not reported, so a comparison with other studies or natural systems cannot be made. The authors make the point that mass transfer limitation is not an issue in the beginning of biofilm development. However, the relatively low fractionation value of 13-17 permil (page 82, line 1) is far from the usually encountered range for fractionation with a maximum of 29 permil which points to possible external mass transfer limitation right from the beginning of biofilm growth in the incubators, probably due to very low velocities (?) and thick diffusive boundary layers. The reported fractionation value seems especially low knowing that it was mea-

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sured relative to bulk DIC. When in the beginning of biofilm growth, the only C-species used was $CO_{2(aq)}$, which will have a lower δ^{13}C than bulk DIC by probably almost 8 permil, then the actual fractionation from C-source to biomass must be even less! Additional ("biofilm-internal") mass transfer limitation due to increasing biofilm thickness may still play a role and cause increasing δ^{13}C-values during later biofilm growth. On the other side, external mass transfer limitation seems unlikely in the light of the pH profiles and the lack of an effect of flow rate. So, why such a low fractionation, even in the initial phase of biofilm growth? A mechanism possibly creating this pattern is a substantial amount of heterotrophic activity, which may also be important in freshwater biofilms. Seen from this point of view, the observed bulk biofilm δ^{13}C values can be interpreted as a product of three processes balancing each other: (1) external and (2) internal mass transfer limitations, and (3) heterotrophic activity. The first is probably really unimportant in the low flow systems studied. While internal mass transfer limitations seem to be the major process in freshwater biofilms, potentially overriding the effect of heterotropic activity, the opposite may be true for marine biofilms.

Technical corrections

page 70, line 4: I guess "which" refers to the thickening of biofilm, therefore replace "which are" by "which is".
page 70, line 6: Perhaps write "dissolved CO_2 " or "$CO_{2(aq)}$ " to make the argument clearer.
page 70, line 10: Maybe better "trend" instead of "change".
page 70, line 17-18: What is the meaning of the second part of this sentence? Replace "originates" by "originate". Maybe it is wise to clarify light as the only ultimate energy source in these phototrophic systems.
page 71, line 3: Replace "fresh water" by "freshwater" as in rest of manuscript.
page 71, line 6: Replace "freshwater-" by "freshwater".
page 71, line 7: Delete "mostly".
page 71, line 12: Delete "therefore".

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page 71, line 14: Delete ",".
page 71, line 15: Maybe mention expected δ^{13}C of DIC from literature.
page 71, line 29: Delete "mostly".
page 72, line 3: Why subscript p for ϵ?
page 72, line 9: "Incorporated δ^{13}C" is misleading, replace by "biomass δ^{13}C".
page 72, line 13: Replace "phytoplankton ranges δ^{13}C" by "phytoplankton δ^{13}C ranges".
page 72, line 17 and 19: Replace "within" by "in".
page 73, line 2: Maybe replace "discuss possible roles of" by "identify".
page 73, line 3: Replace "photosynthetic" by "phototrophic".
page 73, line 6: Replace "special" by "specially".
page 73, line 14: "freshwater".
page 73, line 16: Replace "diatoms" by "diatom".
page 73, line 17: Delete ",". Replace "commerical" by "commercially".
page 73, line 23: Delete "a" from "followed by a continuous".
page 73, line 27: Replace "trough" by "through".
page 74, line 1: Replace "of" by "off".
page 74, line 11: Replace "slide" by "slides".
page 74, line 12: Delete ",".
page 74, line 16: "Germany".
page 74, line 17: Replace "gasflow" by "gas flow".
page 74, line 20: Replace "in the" by "of".
page 75, line 1: Replace "was" by "were".
page 75, line 4: Delete "rate". Replace "per phase was calculated" by "was calculated for each growth phase".
page 75, line 6: Insert "to" after "according".
page 75, line 9: Replace "as the difference of" by "from". It is unclear what the word "sampling moments" refers to and how the average δ^{13}C of DIC was calculated. Is it the average δ^{13}C of DIC of the last 2 sampling moments?
page 75, line 11: The two letter subscript is confusing, just using b_t instead of b_{tn} will

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suffice.
page 75, line 23: Delete “the” from “with the biofilm”.
page 75, line 25: Replace “illumination” by “irradiance”.
page 76, line 9: Replace “micro sensor” by “microsensor”.
page 76, line 19: Replace “describes” by “describe”.
page 76, line 25: Replace “was” by “is”.
page 77, line 16: Replace “with” by “from” or “of”.
page 77, line 19: Refer to Fig. 2a-d only.
page 77, line 22-23: Replace “highest at” by “highest during”. Replace “where after” by “whereas thereafter”.
page 77, line 26: Refer to Fig. 3a-d only.
page 78, line 2: “freshwater”.
page 78, line 6: What is the meaning of the word “differential” in this context?
page 78, line 7: What is meant by “run” here and in the following text?
page 78, line 8: Delete “rate”. Replace “per phase” by “for each growth phase”.
page 78, line 9: Replace “relative to” by “compared to”.
page 78, line 14: Replace “did not significantly differ” by “did not differ significantly”.
page 78, line 23: What does “its” refer to? The pH? Probably replace by “residence time of the medium in the incubator”.
page 78, line 25: “δ^{13}C”.
page 79, line 2 and line 5: Delete “in the incubator”.
page 79, lines 8 and 9: Which “effect of light” are the authors referring to? The clustering of data from various irradiance treatments?
page 79, line 12: “freshwater”.
page 79, line 15: Replace “view” by “trend”. Replace “per” by “for each”.
page 79, line 20: The pronounced increase in pH under higher irradiance in Fig. 6 is hard to recognize since y-axes do not scale equally. Or does this sentence refer to Fig. 5 and the pH of the medium?
page 79, line 23: “decreased again” and/or remained stable?

page 79, line 27: Replace “preformed” by “performed”.
page 80, lines 12-13: Fig. 7b shows HCO_3-consumption to peak in the top of the biofilm, while in that region almost no CO_2 was consumed. This is quite the opposite statement from the text.
page 80, line 20: Delete “total”.
page 80, line 25: Perhaps replace “varied” by “increased”.
page 81, line 7: Delete “under” or change word order.
page 81, lines 11-12: Please report results of correlation analyses in text or in Fig. 1.
page 81, line 23: Delete “the concentration of” and replace “is” by “are”.
page 82, lines 10-13: Include references to graphs supporting this arguments (Fig. 2? Fig. 4?).
page 82, line 14: Insert “at” after “determined”.
page 82, line 22: What is “linear” flow? Should it be “laminar”?
page 83, lines 3-6: The sentence starting with “In natural systemsĚ” seems to be a little off the context.
page 83, line 8: Replace “an opposite trend” by “opposite trends”.
page 83, line 9: How do the 7 permil fit to the δ^{13}C values reported for DIC in freshwater and marine water on page 78, line 6?
page 83, line 11: Isn’t it “must be due toĚ” rather than “was explained byĚ”?
page 83, line 13: Replace “explains” by “explain”.
page 84, line 6: Replace “heterotrophy level” by “level of heterotrophy”.
page 84, line 10: Replace “photosynthetic” by “phototrophic”.
page 84, line 18: “freshwater”.
page 84, line 23: Replace “value” by “values”.
page 85, line 5: Replace “aglae” by “algae”.
page 87, line 6: Replace “WolfGladrow” by “Wolf-Gladrow”.
page 87, lines 11 and 12: Replace “biphospahte” by “biphosphate”. Replace “carborxylase” by “carboxylase”.
page 87, line 21: Replace “Cyprinus carpio” and “Potamogeton pectinatus” by “*Cypri-*

nus carpio” and “*Potamogeton pectinatus*”.
page 88, line 4: Replace “off” by “of”.

Table 1: Delete “in the incubator” in the legend. Include flow velocities here.

Table 2: I guess ND means “not determined”. Why are certain values ND? What do the numbers 4 and 15 in the flow column mean (“average” rows)? Center percentage values to improve readability.

Fig. 1: The legend says “Average maximum growth rates”, but the y-axis has “relative growth rates at 50% absorbance. What are the units on the y-axis? What statistic is used for the error bars? What was averaged, replicates or flow rate treatments? It is hard to extract usable information from this graph: It is virtually impossible to assign error bars to the various treatments. A small offset on the x-axis to separate symbols and error bars in the x-direction could help. This will be ok since only 4 clear irradiance treatments are on the x-axis. Probably two line styles will also improve the graph (marine / freshwater). Or don’t mix freshwater and marine at all (since temperature treatments cannot be directly compared anyway!).

Fig. 2: Replace “biomass per treatment” by “biomass for each treatment” in the legend. Why use upper case letters for the individual graphs, but lower case in the legend (true for all following composite graph panels)? It is not completely clear whether the data shown is bulk biomass δ^{13}C or the δ^{13}C of newly formed biomass. The font size and symbol size are too small, on a print no difference between symbol types can be seen.

Fig. 3: see comments for Fig. 2. Where is the data for the 120 μmol photons m^2 s^{-1} treatment in graph (e)?

Fig. 4: The symbols used for the various treatments are now different from Fig. 2 and Fig. 3 which doesn’t help interpretation. Replace “per irradiance” by “for each irradiance” in the legend. Replace “phases throughout the development” by “phases of development”. What is meant by “successively”? What is the sample size for each

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treatment? Why is data from various treatments / growth phases completely missing?

Fig. 5: Inconsistent use of symbol types when compared with previous figures. Put "respectively" at end of sentence (twice in legend). What is shown in graph A, what in graph B? Probably the three sampling occassions can be denoted in this graph, as well as the pH of the renewed medium (by a line maybe).

Fig. 6: Graphs are very hard to compare because axes scale differently.

Fig. 7: Replace "thick" by "thickness" in the legend. I think the two lines for CO_2 and HCO_3 in graph B are mixed up. What does the abbreviation COD stand for?

References

Finlay, J. C., M. E. Power, and G. Cabana 1999. Effects of water velocity on algal carbon isotope ratios: Implications for river food web studies. Limnology and Oceanography 44: 1198-1203.

Hecky, R. E. and R. H. Hesslein 1995. Contribution of benthic algae to lake food webs as revealed by stable isotope analysis. Journal of the North American Benthological Society 14: 631-653.

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