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August 19, 2021.

Aninda Mazumdar

Editor

Journal Biogeoscience,

Dear Dr. Mazumdar:

We are writing in connection to the Research article: An analysis of the macroalgal $\delta^{13}C$ variability in the Gulf of California (bg-2021-50R1).

We attached a letter with the point-by-point responses to the Reviewer' comments. Each conscientious comment was considered to improve our revised version, update in the web page.

Hopefully, you are agreeing that our revised manuscript has the scientific merits and high standards to be published in the prestigious Journal Biogeoscience

We appreciate all your support on this submission, best regards.

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Referee #3: Michael Roleda

The $^{13}C/^{12}C$ ratios (= $\delta^{13}C$) are indicative rather than definitive proxy of carbon use physiology (Giordano, Beardall & Raven 2005).

R. Right. The term "proxy of carbon use physiology" were replaced by "indicative of the presence or absence of carbon concentration mechanisms (CCMs)".

$\delta^{13}C$ values can vary depending on several variables such that inconclusive $\delta^{13}C$ signatures have been reported in several seaweed species (See Roleda and Hurd 2012, page 412).

R. Right. The isotopic signature evidenced the activity of CCM, but it is inconclusive about the preferential uptake of HCO_3^- and/or CO_2 in photosynthesis. Many macroalgae genus and species showed a large $\delta^{13}C$ variability ($-10<\delta^{13}C>-30‰$) in our study. 84% of the total analyzed specimens were classified under strategy 2, using both HCO_3^- and/or CO_2. In agreement with the Reviewer's comments, complementary techniques to the isotopic tools are required. The preferential DIC uptake of macroalgae can be assessed by pH drift experiments (Hepburn et al., 2011; Roleda and Hurd, 2012; Fernandez et al. 2014, 2015; Narvarte et al., 2020) and/or by simultaneously measuring the CO_2 uptake and O_2 production rates using membrane-inlet mass spectroscopy (MIMS) (Douchi et al., 2019; Burlacot et al., 2020).

However, several genus and species showed $\delta^{13}C$ values reflecting DIC uptake sources. For example, specimens belonging to 58 species showed carbon uptake strategy 1 that use only HCO_3^- ($\delta^{13}C>-10‰$). Also, $\delta^{13}C$ values lower than -30‰ that denote uptake of CO_2 by diffusion (strategy 3) were observed only in Rhodophyta *Schizymenia, Halymenia,* and *Gigartina*.

In this study, were there any widely distributed species collected?

R. Most of the macroalgae species showed a limited distribution along the Gulf California coastlines. Few cosmopolites' species included *Colpomenia tuberculata*, *Sargassum sinicola, Padina durvillae,* and *Ulva lactuca*. This was clearly stated in the

revised version (lines 250-251: “Few cosmopolites’ species included *Colpomenia tuberculata, Sargassum sinicola, Padina durvillei,* and *Ulva lactuca*.”)

Did the $\delta^{13}C$ varied with collection sites (tidal level and latitude), season, and collection years?

R. Yes. $\delta^{13}C$-macroalgal varied with collection sites (tidal level and latitude), season, and collection years (lines 371374: “Multiple comparison analysis of $\delta^{13}C$ signals evidenced significant differences between the most common genus and species of macroalgae between and within assemblages grouped by coastal sector, season and collecting year (Supplementary Information Tables SI-2-3).”). For example, genus *Padina*, *Ulva*, and Codium showed differences in the $\delta^{13}C$ signals related to sites and seasons but non-indicative of changes in the carbon use physiology.

For some key species, the $\delta^{13}C$ data could have been backed up in combination with other techniques such as pH drift experiment, and/or HCO_3^- utilization pathway inhibitors (Fernandez et al. 2014, 2015), which are relatively easy to do, to categorically establish their carbon use physiology. For example, $\delta^{13}C$ in combination with pH drift experiments, HCO_3^--using macroalgae can shift seawater pH to 9.0 or higher and will have corresponding $\delta^{13}C$ values less negative than -30‰. Conversely, CO_2-using species have $\delta^{13}C$ values more negative than -30‰ and will be unable to raise pH above a critical value of pH 9 (Maberly et al. 1992). Unfortunately, this may not be possible anymore. Otherwise, additional data will make this paper better- be critical on the use, significance, and limitations of solely using δ13C as a proxy for carbon use physiology.

R. Right. This comment was considered. Please see Lines 683-698: “Measurements of $\delta^{13}C$ signals evidence the presence or absence of CCMs in macroalgae and are indicative of carbon use physiology (Giordano et al., 2005), however, the isotopic signature may be inconclusive in the determination of the efficient use of one or more DIC species (CO_2 and/or HCO_3^-) (Roleda and Hurd, 2012). The preferential DIC uptake of macroalgae is assessed by pH drift experiments (Hepburn et al., 2011; Roleda and Hurd, 2012; Fernandez et al. 2014, 2015; Narvarte et al., 2020) and it can be determined by

simultaneously measuring the CO_2 uptake and O_2 production rates using membrane-inlet mass spectroscopy (MIMS) (Douchi et al., 2019; Burlacot et al., 2020). Macroalgae that are unable to raise the seawater pH>9.0 are primarily CO_2-users, while those that can raise the seawater pH>9.0 (absence of CO_2) are HCO_3^--users (Roleda; Hurd, 2012). Those differences in the carbon uptake strategies can be easily deduced by pH drift experiments, which were not done in our study but reported in the literature (Supplementary Information Table SI-4). Also, the change in $\delta^{13}C$ signature within the range specific to a carbon use strategy (e.g., mix HCO_3/CO_2^--user) can be complemented by simultaneous measurements of O_2 and CO_2 produced and consumed, respectively during the photosynthetic using MIMS. For example, photosynthetic O_2 production in a certain macroalgae species with an active CCM preferring (e.g., CO_2) is about ten times higher than no active CCM (Burlacot et al., 2020)."

When additional data is not possible, the authors are encouraged to give emphasis on the limitations of the study. Despite the huge dataset and corresponding statistical analyses, the significant correlations are meaningless when they do not have physiological and ecological relevance. For example, why would morphology determine carbon use physiology? Is there a specific morphology that would tend to be strictly CO_2-user rather than mix HCO_3^-/CO_2-user or strictly HCO_3^--user?

R. Right. The limitations of our study were emphasized in the Conclusions (Lines 792-808): "Despite the large dataset and corresponding statistical analyses, our study faces limitations due to research design and because no research on $\delta^{13}C$-macroalgal analysis was developed previously in the GC. The primary deficiency is the lack of pH drift experiments to discriminate $\delta^{13}C$ signal variations to the carbon uptake strategies to determine preferential DIC uptake of macroalgae (CO_2 or HCO_3^-). The second limitation concerns the lack of controlled experiments to discern what type of CCM is expressed in macroalgae (e.g., direct HCO_3^- uptake by the anion-exchange protein AE, types of mitochondrial AC, or the co-existence of different CCMs). Also, more research is required to assess the biological or ecological relevance of the $\delta^{13}C$ variability in function of the morphology (e.g., DIC uptake efficiency and isotope discrimination during carbon assimilation and respiration). Future studies assessing the ability of macroalgae to use

CO_2 and/or HCO_3^- can be assessed by pH drift experiments and/or MIMS in the cosmopolites' species and within of genus with differences in the $\delta^{13}C$ values between species (e.g., Ulva and Sargassum). Finally, controlled experiments in laboratory and mesocosm type combined with field studies are required to elucidate what type of CCM is expressed in macroalgae. Even so, the $\delta^{13}C$-macroalgal was a good indicator to infer the presence or absence of CCM's and identify the macroalgae lineages that could be in a competitive advantage based on their carbon uptake strategy and identify their geographical distribution along with GC."

Environmental conditions may change $\delta^{13}C$ values but should not in that instance change carbon use physiology, which is most likely inherently species-specific. A change in δ13C signature within the range specific to a carbon use strategy (e.g. mix HCO3/CO2-user) may indicate the presence of the CCM capacity of algae but may not indicate preferential uptake of certain Ci species (HCO_3^- or CO_2), which could be measured using membrane-inlet mass spectroscopy (MIMS).

R. Right. The carbon use strategy is inherently species-specific. The environmental conditions modulate the δ13C values but do no change the carbon use physiology. Please, see Lines 683-698 and 792-808.

In extreme cases, some species have been reported to have totally different δ13C values representing different carbon use strategy. Can these cases be attributed to incorrect species ID from different studies? In this regard, correct species ID is of utmost importance specially those cryptic and morphologically simple but phenotypically plastic taxa. These species may require molecular ID. How well was Ulva discriminated based on morphology? See Roleda and Heesch 2021 Food Chemistry and references therein on problems related on Ulva ID. The Ulva LPP (linza-procera-prolifera) complex is another issue to contend with (Shimada et al. 2008, Herrero et al. 2020).

R. Reviewer's comments are correct. The genus *Ulva* include species morphologically simple but phenotypically plastic taxa. In our study, we considered that the identification

of macroalgae species was correct. However, molecular identification should be considered in future studies.

Throughout the manuscript, the authors should be clear whether the increase/decrease, change, or variability in $\delta^{13}C$ refers to change in carbon use? Or shift within a specific carbon use strategy. Then what is the significance of this shift or variability? How does it explains lines 683-685: "Filamentous uniseriate and pluriseriate with erect thallus and C-Tubular) and genus (e.g., Colpomenia, Padina, Polysiphonia and Gracilaria) revealed that certain life forms are better monitors explaining the variability of $\delta^{13}C$-macroalgal (and $\Delta^{13}C$ values) than others." It is not convincing that there is biological and ecological basis or support that δ13C variability and therefore carbon use physiology is controlled by morphology. For example, within the genus Halimeda, different species (relatively the same morphology?) measured different carbon use physiology based on pH drift experiment (Narvarte et al. 2020).

R. Right. Please see Lines 735-740: "The $\delta^{13}C$ variability in morphological groups refers to change within a specific carbon use strategy, but not change in the carbon use physiology that is inherently species-specific. The biological or ecological relevance of the $\delta^{13}C$ variability in function of the morphology, in terms of the efficiency in the use of DIC and the isotope discrimination during carbon assimilation and respiration, must be investigated in species of same genus morphologically different or between same morphological structures belonging to a different taxon."

The title can be made more interesting. So, what does this variability suggest?

R. The title was updated to: "An analysis of the variability of δ13C in macroalgae from the Gulf of California: indicative of carbon concentration mechanisms and isotope discrimination during carbon assimilation"

Specimen were collected during spring (March-April) and dry season (nominally from November to May) from 2008 to 2014. When were the environmental parameters measured? E.g. collected only during the sampling? For example, was

the pH measurements snapshot? Or daily average? Does the temperature category represent daily average or seasonal average? Is there interannual variations?

R. Environmental parameters were measured in each ecosystem during the sampling surveys (1-2 whole days per survey, 4-5 surveys per year). All parameter data were grouped by season and year. Several parameters, including temperature, pH, and salinity, showed differences between seasons and coastal sectors in the Gulf of California. Non-analysis of interannual variations were conducted because of our limited dataset.

What does "composite samples" means? For each species (big or small), was the whole plant (rhizoid/holdfast, stipe, and blade) analyzed as one unit? Please specify. Otherwise, what are the implications of analyzing only parts or the whole plant.

R. We referred to "composite samples" because 4-5 macroalgae specimens of the same species (whole plant), collected at the same site and time, represented a sample of the 809 samples analyzed. The thallus or phyllodes (only for *Sargassum*) of the 4-5 specimens were used for the isotopic analysis. The thallus is whole macroalgae.

How different is the 4th strategy of DIC uptake from the other three? Have not encountered this classification before (please cite a reference). Is this only for calcifying species?

R. Strategies 1 and 4 are in the same $\delta^{13}C$ ranges. Both are HCO_3^- -users. However, based on the categories of Díaz-Pulido et al. (2016), calcifying macroalgae species, with a different carbon uptake strategy influenced by the calcifying process, are categorized under strategy 4.

What type of calcification? For example, were both *Amphiroa* and *Padina* classified under the same category?

The calcification mechanisms in the calcifying macroalgae are diverse and species-specific, which are out of our study scope. In this case, the articulated coralline red algae *Amphiroa* and the calcifying brown alga *Padina,* classified in the same strategy 4 as HCO_3^- -users, shows differences in theeir calcification mechanisms. *Amphiroa* deposits mostly aragonite both extra-cellularly between frond layers and intercellularly within the

cell wall matrices (Kraft et al. 2004). While *Padina* form lightly calcified fronds made of aragonite deposited externally in a semi-enclosed space formed by infoldings of the margin of the thalli (Okazaki et al., 1986; Raven et al. 2002; Enríquez & Rodríguez-Román 2006).

Line 711: "CO2 carbon mechanism"?

R. It was a finger mistake. The correct is "CO_2 concentrating mechanisms…"

The English language can still be significantly improved.

R. The English language was revised, and now the paper is in the correct English.