10 September 2019

RE: Revisions to manuscript

Dear Editor, Dr. Woulds,

Please find attached our revised submission to *Biogeosciences* entitled "**Validation of carbon isotope fractionation in algal lipids as a *P*CO_2 proxy using a natural CO_2 seep (Shikine Island, Japan)**" by Caitlyn R. Witkowski, Sylvain Agostini, Ben P. Harvey, Marcel T.J. van der Meer, Jaap S. Sinninghe Damsté, and Stefan Schouten. We would like to thank the editor and reviewers for their thoughtful and constructive criticism, which has resulted in an improved manuscript.

In the comments by the reviewers, most issues were primarily on details or technical. Some of these comments were the need to include more information or clarification on the site conditions (e.g. occurrence of extreme weather, typical temperatures, *P*CO_2 measurements). A major comment which we addressed is the other potential influences on the *P*CO_2 reconstructed from these potential proxies. We hope that we have adequately addressed their concerns, which we do in detail below.

Sincerely,
On behalf of all co-authors,

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Detailed Response to Reviewer comments

Reviewer 1

Witkowski et al. present a very interesting dataset that suggest that we may be able to use general algal biomarkers for reconstructing past PCO2. My impression of the MS is very positive although it is clear from the results that still more work needs to be done. It is very well organised and easy to read. The date is well presented and the interpretations are sound. I congratulate the authors on their effort. I think that the manuscript should definitely be published in BG and have no major critics. However, I do have a number of specific/technical comments (listed below) that I hope will help to improve the final revision of the MS.

We thank the reviewer for the comments, as well as for the recommendation for publication. Below, we respond to the specific/technical comments expressed by the reviewer.

Page 3 Line 9. Suggest placing a reference to figure 1 here.

We have placed a reference to Fig 1 here.

Page 3 Lines 15-19. Why were the currents and winds measured in 2014 and 2015 and not in 2016 when the samples were collected? How comparable is this for the 'normal' situation in this region?

The currents and winds were studied in detail during the 2014-2015 expeditions (Agostini et al., 2015). Unpublished observational data suggest that the observed currents and winds are normal for the region, based on visits to the site on a monthly basis for the past 5 years. Although 2016 was a particularly strong season for storms, this region experiences these kinds of storm activity annually.

Page 3 Line 25. Is the abbreviation SPM properly introduced?

We have removed all mention of SPM, as these samples did not yield enough organic material for isotopic measurements.

Page 4 Line 8. Remove 'then'.

We have removed 'then'.

Page 4 Lines 10-11. Change to '..... NBS-19), flushed with He, injected with 500 µL of 85% H3PO4, and reacted for 1 h.'

We have changed this phrasing.

Page 4 Lines 11-12. Change to 'The headspace was measured and average values and standard deviation errors reported are based....'

We have changed this phrasing.

Page 4 Lines 16-17. Change to '.... using ultrasonication (5 times) with 2 ml dichloromethane (DCM): MeOH (9:1 v/v).'

We have changed this phrasing.

Page 4 Lines 19-20. Change to '.....and the organic matter the DCM layers were pooled and dried over Na2SO4.'

We have changed this phrasing.

Page 4 Line 20-21. Change to 'The resulting hydrolyzed TLEs were eluted over an alumina packed column and separated into apolar....'

We have changed this phrasing.

Page 4 Line 22. Remove 'then'

We have removed 'then'.

Page 4 Line23-24. Change to '.....prior to analyses by gas chromatography-with flame ionizationdetection(GC-FID),GC-massspectrometry(GC-MS),andGC-isotope-ratio mass spectrometry (GC-IRMS).'

We have changed this to "analyses".

Page 4 Line 25. Would it not better to report that GC-FID was used for quantification and to check the signal to noise ratio?

We have changed this phrasing.

Page 4-Line 26. What are the ideal concentrations? What is the range?

We have added that ca. 1 ug of polar fraction was injected on-column.

Page 4 Line 28. Change to '.... and He is used as carrier gas.'

We have changed this phrasing.

Page 4 Lines 28-30. Suggest changing it to 'The GC oven was programmed from70◦C to 130◦C at 20◦C/min and then to 320◦C at 4◦C/min at which it was held for 10 min.'
We have changed this phrasing.

Page 4 Line 34. Replace 'C20 and C24' with 'the same'.
We have changed this to 'the same'.

Page 5 Lines 6-9. Why include this information again? You have already given this information in the method section.
We used this paragraph as a summation of information that was spread across the site and materials subsections of the methods. Although we agree with the reviewer that there is some repetition, we think it is important to include in both sections.

Page 5 Lines 5-11. If the SPM samples were not included in this study why mention them at all? See no reason for this and suggest removing all information related the SPM samples.
We have now removed all mention of SPM.

Page 5 Lines 12-13. I cannot find the supplementary information anywhere so cannot comment on this figure.
Thank you for pointing this out. We have now included the supplementary material.

Page 5 Lines 17-20 +Fig 2. Not all compounds mentioned here are clearly labelled in Fig. 2. For completeness this should be corrected.
We have not specifically indicated all compounds as they crowd the chromatograms and are not part of the study. Our point was merely to show that the compounds investigated are abundant, well-separated, and that there are no large differences between sites.

Page 5 Line 23 and onwards. Considering that only two (or three) sites are compared it is incorrect to talk about 'change' here (or shift in the next lines). It would be better to report the 'differences' between the sites or, as a couple of lines later, mention if the values are higher or lower if compared to....
We have changed this phrasing to 'differences' from 'change'.

Page 6 Line 27– page 7 iine 3. Here the possibility of a contribution of terrestrial derived cholesterol is discussed. I agree that this cannot be completely excluded but was wondering if the authors have some

more information about the relative terrestrial contributions to the sediments in this region. Looking at fig 2, for instance, suggest that there is no substantial presence of terrestrial HMW n-alkanols. What about biomarkers present in the other fractions obtained?

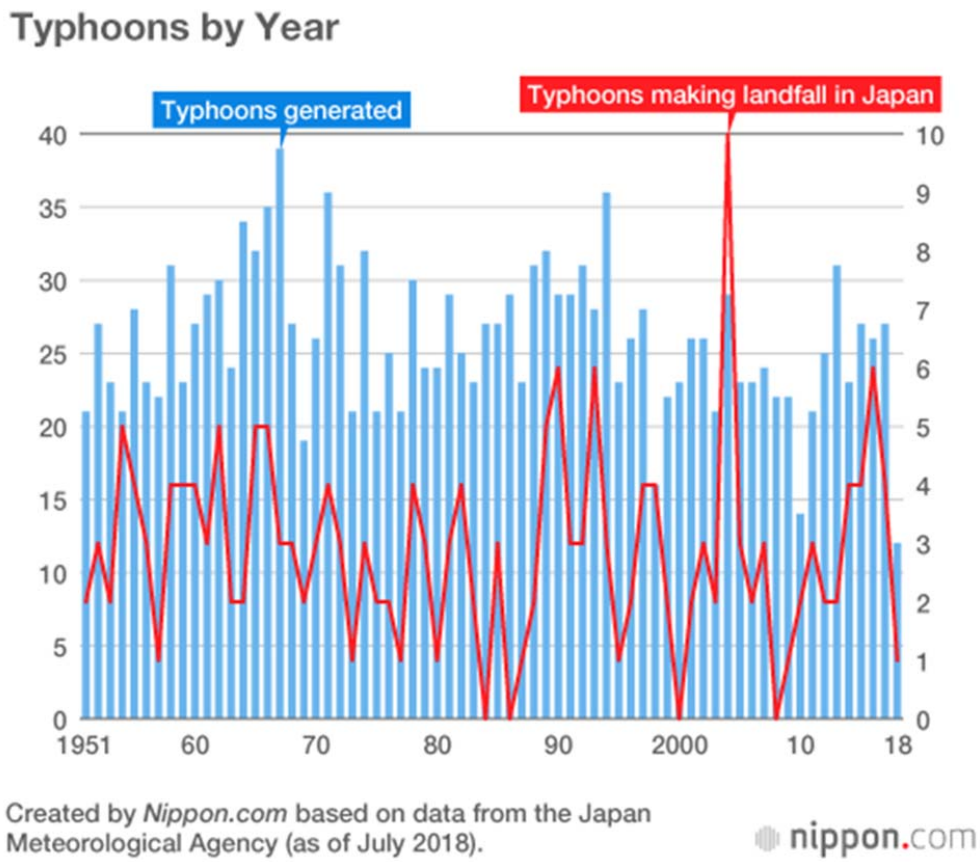
We have added a sentence here that explains that the samples lacked triterpenoids and long-chain alcohols typical of higher plants, suggesting a low amount of terrestrial input.

Page 7 Lines 15-25. I find this a bit of a confusing section, particularly in line with the information reported in the method section 2.1. As mentioned earlier I do not understand why the currents and winds were measured in 2014 and 2015 and not in 2016. It now seems that the conditions between the sampling seasons were completely abnormal. In addition, would it be possible to add a few references to information given in this section. I assume that the kind of impact this had on the corals etc must have been properly documented.

Based on unpublished observational data from part of the co-authors visiting the site on a monthly basis, the conditions covering 2014-2015 are typical of what they've witnessed over the past six years of research. This study on winds and currents is intensely time-consuming and was thus not repeated in 2016.

As commented above, typhoons and strong tropical storms are common in this region and occur on an annual basis. Our June collected samples showed lower reconstructed values for pCO_2 than September, which may be explained by this annual storm season which mixes the surface sediments of the bay every year. Our sampling year (2016) happened to have particularly intense storms reaching land (see supporting figure attached here).

We have added a sentence in Section 2.1 that states, "Monthly surveys in the bays over the past five years show that these sites have similar annual mean values for temperature, salinity, and currents. Weather station data shows that severity of seasonal extreme weather, e.g. typhoons, varies on an annual basis (Japan Meteorological Agency)."



Page 8 Line 4. It should be ' Witkowski et al. (2018)'

This is changed to Witkowski et al. (2018).

Page 8 Line 6. Change to '....however, has never been determined.'

This is changed to '…however, has never been determined.'

Page 8 Line 17. Change to '.....sites for all three....'

This is changed to 'for'.

Page 8 Line 23. Change to '....as it is the only....'

This is changed to 'as it is the only'

References. Please check all references carefully. It should be 'Sinninghe Damsté, J. S.' and not 'Damste, J. S. S'.

We have checked the references more carefully.

Figure 3 and 4. Currently the data in these figures is presented as line plots. However, considering that we are only dealing with samples from three discrete areas I feel that this is misrepresenting the results suggesting that there are trend between the three sites. Suggest removing the lines, showing the results as individual points.

We have removed the lines and show the results as individual points.

Reviewer 2:

Witkowski et al. report carbon isotope fractionation from CO2 into algal lipids found in various sample substrates in the vicinity of natural CO2 seeps. They successfully use these sites to ground-truth the use of algal lipid carbon isotope fractionation as a pCO2 proxy. I congratulate the authors on this novel, comprehensive, and concise study. I have some minor comments that should be addressed before acceptance. Furthermore, I would like to ask the authors to use continuous line numbers in the future, as is standard practice.

We thank the reviewer for the recommendation for publication and comments which have improved the manuscript. The reviewer had asked us in a separate comment for a clarification on two comments in our rebuttal, which we have included under the corresponding original questions (Comments on Page 4, 1 and on Page 5, 6-8).

Line comments:

…I would like to ask the authors to use continuous line numbers in the future, as is standard practice.

We used the Biogeosciences format which specifies using the numbering shown in this manuscript (though we also prefer continuous line numbering).

Page 4, 1: Why were the filters combusted only at 300 C for 3h? Standard practice is 450 C for 5h or similar.

We thank the reviewer for bringing this to our attention. Upon further investigation, we have found that the standard procedure used in our lab (and thus used in this study) was 450◦C for 4 h. Our apologies for the confusion.

Page 5, 6-8: Unclear if the reported pCO2 values (is this dissolved CO2?) are taken from the literature or are original data. If these are original data, the authors need to state in detail how pCO2(aq) was calculated. If these are literature values, and not measured from the same samples as the d13C-DIC values, the authors need to state why they consider these values to be adequate for comparison with their samples (both in a spatial and temporal sense).

The pCO_2 data presented in the former studies were calculated using the carbonate chemistry system analysis program CO2SYS using the measured values for pH_{NBS}, temperature, salinity, and total alkalinity (TA) values. There is indeed high variability both temporally and spatially. On Page 3, Line 11-12, we include the standard deviations (Control pCO_2 309 ± 46 µatm, Mid pCO_2 ca. 460 ± 40 µatm, and High pCO_2 769 ± 225 µatm). In Figure 5, these standard deviations are included as horizontal error bars where the "Actual pCO_2" values measured at the site lie on the x-axis.

Based on the reviewer's comment, we have now added a few lines to the discussion in 4.3 regarding possible cause for pCO_2 underestimation in our mid and high sites, in which we state that this variability could have major impacts on the reconstructed values, as these algae are exposed to different levels of pCO_2 even within the same site.

Page 5-6: The authors should include all data as either a main text table or supplementary table/data file, containing d13C-DIC, d13C-CO2, d13C of biomarkers etc.
We have included three supplementary tables, one with $\delta^{13}C$ of all the measurements taken, one of the calculations used to derive a corrected $\delta^{13}C$ of CO_2, and one with ε_p calculations to estimated pCO_2 for all three biomarkers.

Page 8, 11-12: Is it reasonable to assume a constant temperature? Is there no seasonality in primary productivity at this site?
Here, we use an annual average temperature because the surface sediments are an integrated accumulation of all primary productivity over the year. Although primary productivity is higher in the spring and summer, this site has some (observational) productivity throughout the year.

Page 8, 25-Page 9, 21: Here you could discuss the recent paper by Badger et al. (Climate of the Past, doi. 10.5194/cp-15-539-2019) suggesting insensitivity of alkenone13C at low-mid pCO2 levels.
In the discussion 4.3, we have added "There are several possible explanations to why there is an underestimation. As discussed above, carbonate concentration mechanisms may be operating in a large number of phytoplankton, such that they become relatively enriched in 13C and thus lead to lower reconstructed PCO2 values (Badger et al., 2019; Stoll et al., 2019)." We have also added the possibility of a variable b value.

Page 9, 18: "annually"
This is changed to "annually"

Page 9, 28: I would suggest being more cautious with the wording ("likely") here. Can you provide evidence to support your argument for allochthonous input? Where would this come from?
We use the word "likely" here as we do not have independent support for the input of allochthonous organic matter. That material would come from surface sediments transported from the edge or outside of the bay where CO_2 levels are much lower than near the CO_2 seep. Since this is not a very large distance (500 meters) we can imagine that strong circulation events like typhoons

would resuspend surface sediments and transport them to near CO_2 vents. In the conclusion, we have added, "from nearby sediments deposited under normal PCO_2 levels caused by the intense annual typhoon activity in this region."

Reviewer 3:

The authors of the manuscript use natural CO2 seeps in the vicinity of Shikine Island(Japan) to investigate the relationship between different concentrations of aqueouspCO2 and carbon isotope fractionation in three organic compounds extracted from surface marine sediments, diatoms, plankton tow, and microalgae. It is a novel approach that utilizes a unique natural setting. The subject of the manuscript fits well within the scope of the journal, and the results of this project would certainly be of significant interest to paleoceanographers and paleoclimatologists who use carbon isotopic composition of biomarkers as a proxy for pCO2. The manuscript, however, contains several major and minor issues that need to be addressed before the manuscript is considered for publication.

We thank the reviewer for the comments and recommendation for publication. Below we will respond to each of the comments, which will improve the manuscript.

MAJOR ISSUES

FIRST, the choice of organic compounds (biomarkers). The authors need to provide a clear rationale as to why loliolide, phytol and cholesterol were chosen for this work. None of these compounds can uniquely be linked with a source (i.e. they can come from a variety of sources including marine and terrestrial), so it is not clear how applicable their work (assuming these compounds are targeted) would be to paleo studies. In fact, the problem with significant underestimation of reconstructed pCO2 (see the next issue below) might be due to a poor control of what those compounds actually represent in terms of the source in this study.

We chose these organic compounds because they actually are representative of a wide range of producers. As outlined in the introduction, our aim is to explore the suitability of biomarkers from multiple sources for PCO2 reconstructions, as previously done for porphyrins and phytane. By exploring a larger groups of producers in open ocean settings, we may be able to extend the PCO2 record derived from epsilon p, as has been done for the Cretaceous (Bice et al., 2006; Sinninghe Damsté et al., 2008; Naafs et al., 2016) and for the Phanerozoic (Witkowski et al., 2018), both reconstructed from phytol's diagenetic product phytane. Although the sources of these compounds may be both terrestrial and/or marine, in an open marine setting they will almost entirely be derived from phytoplankton (and in the case of cholesterol, also the zooplankton that consume and retain the isotopic composition of these same phytoplankton). Here, we are on the coast of a small island in open ocean and have examined our GC-MS runs for the potential presence of characteristic terrestrial biomarkers to test whether the contributors of these compounds also

include terrestrial inputs from the island. The lack of triterpenoids and long-chain alcohols typical of higher plants suggests that our source signal is overwhelmingly marine.
We added a small section (revised manuscript Page 7, lines 2-14) in the discussion (Section 4.1) to further describe and discuss the sources and why we chose these specific compounds. When these compounds are first described in the results, we make a note in the manuscript that the sources will be discussed in the discussion.

SECOND, underestimation of reconstructed pCO2 Figure 5 and the accompanying discussion show that the reconstructed pCO2 are significantly lower than the measured values at both the Mid and High pCO2 sites by almost a factor of two. The possible reason(s) for this are not really addressed and mainly limited to "some site limitation". This issue requires a more detailed discussion particularly with regard to possible influences of different OM sources and the validity of the assumptions used for calculating the epsilon values for each compound (Section 4.2).
In section 4.3, we reconstruct PCO2 and describe why these reconstructed values are lower than the measured high PCO2 sites, primarily focused on the novelty of using such a site and the further research required. We have discounted different OM sources due to the lack of terrestrial biomarkers.
However, we agree with the referee that several reasons could explain the underestimation and have expanded several sections to further consider alternative hypothesis. First, we expanded the text at end of 4.2 to include why epsilon f (maximum fractionation) is not fully expressed at the high CO2 site, such as species' affinity for carbon concentration mechanisms which utilize 13C-enriched bicarbonate, as well as the recent studies that show different Rubisco types may yield lower epsilon f than previously assumed (Thomas et al., 2018). Second, we expanded the discussion at the end of section 4.3 to raise the possibility of changing b value, which includes factors influencing fractionation other than CO_2 .

MINOR ISSUES
p. 1, line 16, "general algal compounds": What does this mean? Are these compounds sources only by algae?
As per our response in major issues 1, general algal compounds contrast to species-specific algal biomarkers, e.g. alkenones, which occur in limited number of genera. General algal biomarkers refer to compounds that are derived from a multitude of species, i.e. a large part of the phytoplankton community, presumably overwhelmingly from phytoplankton sources based on our analyses. We clarified this in the abstract and the text in the introduction on Page 3, line 10.

p. 2, line 1, "current proxies leave much to be desired, often with large uncertainties and conflicting values": Could the authors elaborate on this, i.e. what specific issues with the current proxies do the authors have in mind and how this work would reduce these limitations?

We have elaborated on this on Page 2, lines 1-10. Although there has been much progress in development of PCO2 proxies, there are few proxies which can span timescales over 100 Ma. The few that can span longer periods are terrestrial biomarkers, which tend to have larger uncertainties, e.g. paleosols. PCO2 reconstructions based on epsilon p has its problems, particularly at lower PCO2 but tends to have smaller uncertainties and so if this could be applied to longer timescales, it would offer a long geological record (less influenced by local carbon cycling) and could help constrain the estimates for these older records.

p. 3, line 25, "SPM": What does SPM stand for?

We have now removed all mention of SPM, as these samples did not yield enough organic material for isotopic measurements.

p. 6, line 14, "lighter d13C values": a d13C value cannot be 'lighter' or 'heavier'. It is a number. Use 'lower' or 'higher' instead.

We changed this to higher (^{13}C enrichment) or lower (^{13}C depletion) throughout the manuscript.

p. 6, lines 18-19, 29-30, "the primarily diatom-limited compound loliolide": It is an very common compound derived from many sources, including macrophytic algae and terrestrial plants, so linking it specifically with diatoms is somewhat risky. Furthermore, this compound is know to be a degradation product of fucoxanthin and other carotenoids, which are also difficult to link to a particular source during paleoreconstructions.

We added a small section to further describe the sources of the biomarkers and, as in response to the first major comment, describe why we chose these specific compounds. We added a small section (revised manuscript Page 7, lines 2-14) in the discussion (Section 4.1) to further describe and discuss the sources and why we chose these specific compounds. When these compounds are first described in the results, we make a note in the manuscript that the sources will be discussed in the discussion.

Regarding the source of loliolide, it is established that it is a product primarily derived from fucoxanthin. Repeta (1988) explores possible carotenoid sources of loliolide in modern sediments and demonstrate the fucoxanthin contributes to loliolide but are unable to demonstrate a parallel

conversion of diadinoxanthin and other carotenoid epoxides to loliolide. Fucoxanthin is found in diatoms, as well as brown seaweeds, and is not common in terrestrial plants. The vast majority of fucoxanthin in the world is derived from diatoms, which make up a vastly larger mass of producers than brown seaweeds and generally contain more than four times as much fucoxanthin as brown seaweeds. Indeed, loliolide is often abundantly found in upwelling regions where diatoms tend to dominate.
We added a sentence to further describe the different potential sources of loliolide. However, given that all the theoretical sources are based on carotenoids, these should have the same biosynthetic pathways and thus should not impact the isotopic composition of the degradation product loliolide.

p. 9, lines17-18, "allochthonous input of sediment": Need to provide more detail here. Is it just about sediment or about are organic matter/biomarker sources with different epsilon values that would make reconstructing pCO2 more complex?
We will add more detail here to describe what we mean by allochthonous input, here referring to the deposit of sediment that contain our organic compounds that has originated at a distance (e.g. outside of the bay) into our elevated PCO2 sites. Sediment mixed between the high PCO2 site and sediment far removed from the seep would likewise mix the epsilon p signal derived from these sediments.

FIGURES
Figure1: The figure is confusing, i.e. it is difficult to know where this island is. It needs to be shown in a broader context, e.g. with a map of Japan at least. Geographic maps also typically have lines of latitude and longitude (shown as grid) along the X- and Y-axes. Also, the direction of the geographic North should be indicated.
We have revised this map to include an insert of the larger region (i.e. Japan) with the location of the island. We have also included latitude and longitude lines on the x- and y-axis, as well as geographic North.

Figure 2: Is it a GC-FID trace or GC-MS (TIC or SI mode, if so which m/z?)? Why not to give the names of the compounds next to the peaks rather than list them in the caption?
This is an GC-FID trace, which is now labelled. We have put the compounds next to the peaks rather than in the caption.

Figure 3: A), B), and C) are not shown on the plots. These need to be labelled. What are the error bars associated with the d13C values shown on the plots? Also, instead of 'Control', 'Mid', and 'High' show the actual pCO2 values.

We added labels for A, B, and C. The error bars are 0.5‰, as described in the text. These were difficult to see in the figure, as they all overlap with one another, but have now been added. Although we agree that actual PCO2 values would be ideal, the large fluctuations of these measured values (as pointed out in early comments) are the reason we prefer to keep the current labels.

Figure 4: Here and in text (p. 8, lines 18-20), explain how the errors associated with the epsilon(p) values were calculated?

We used the standard deviation of the many samples taken at each site, which we have now included in the text.

Validation of carbon isotope fractionation in algal lipids as a PCO_2 proxy using a natural CO_2 seep (Shikine Island, Japan)

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Abstract. Carbon dioxide concentrations in the atmosphere play an integral role in many earth system dynamics, including its influence on global temperature. ~~Long-term trends~~The past can provide insights into these dynamics, but unfortunately ~~though~~ reconstructing long-term trends of atmospheric carbon dioxide (expressed in partial pressure; PCO_2) remains a challenge in paleoclimatology. One promising approach for reconstructing past PCO_2 utilizes the isotopic fractionation associated with CO_2-fixation during photosynthesis into organic matter (ε_p). Previous studies have focused primarily on testing estimates of ε_p derived from the $\delta^{13}C$ of species-specific alkenone compounds in laboratory cultures and mesocosm experiments. Here, we analyze ε_p derived from the $\delta^{13}C$ of more general algal biomarkers~~compounds~~, i.e. compounds derived from a multitude of species, from sites near~~at~~ a CO_2 seep off the coast of~~near~~ Shikine Island (Japan), a natural environment with CO_2 concentrations ranging from ambient (ca. 310 μatm) to elevated (ca. 770 μatm) PCO_2. We observed strong, consistent $\delta^{13}C$ shifts in several algal biomarkers from a variety of sample matrices over the steep CO_2 gradient. Of the three general algal biomarkers explored here, namely loliolide, phytol, and cholesterol, ε_p positively correlates with PCO_2, in agreement with ε_p theory and previous culture studies. PCO_2 reconstructed from the ε_p of general algal biomarkers show the same trends throughout, as well as the correct control values, but with lower absolute reconstructed values than the measured values at the elevated PCO_2 sites. Our results show that naturally occurring CO_2 seeps may provide useful testing grounds for PCO_2 proxies and that general algal biomarkers show promise for reconstructing past PCO_2.

1 Introduction

The current increase in the atmospheric concentration of carbon dioxide (expressed in partial pressure, PCO_2) plays a leading role in climate change (Forster et al., 2007). PCO_2 is significantly higher now than it has been in the past 800 ka (Lüthi et al., 2008) and although long-term changes in PCO_2 are not uncommon over millions of years (Foster et al., 2017), this current spike in PCO_2 has occurred within only the past two centuries (IPCC, 2013). Uncertainties remain on the exact magnitude to which PCO_2 influences climate, as well as the exact response of the environment to these climate changes. Long-term PCO_2

trends help us better understand the context for these changes and are reconstructed via indirect means, i.e. environmental proxies. Two proxies can span timescales over 100 Ma (Foster et al., 2017), e.g. the terrestrial proxies paleosols and leaf stomata. The paleosol proxy has large uncertainties due to the difficulties in constraining soil respiration (Breecker et al., 2010;Cotton and Sheldon, 2012) due to carbon isotopic fractionation during microbial decomposition (Bowen and Beerling, 2004), carbonate diagenesis (Quast et al., 2006), and other local and regional influences on carbon cycles in these terrestrial settings. Although the leaf stomata proxy is often better constrained than paleosols, some experiments do not show the expected trends (Ellsworth et al., 2011; Ward et al., 2013; DaMatta et al., 2016), suggesting that factors other than PCO_2, e.g. ecological systems, species, and development stage, also impact the leaf stomata proxy (Xu et al., 2016). The development of new proxies for PCO_2 may help us better constrain past long-term trends, particularly marine-based proxies which tend to have more homogenized signals but are currently relatively limited in time.

A proxy that has been explored with mixed success over the past several decades is the stable carbon isotopic fractionation associated with photosynthetic inorganic carbon fixations (ε_p), which has been shown to positively correlate with PCO_2 (Bidigare et al., 1997; Jasper and Hayes, 1990; Zhang et al., 2013). ε_p occurs as the CO_2-fixing enzyme in photoautotrophs, Rubisco (ribulose 1,5-biphosphate carboxylase oxygenase), favors ^{12}C which consequently results in photosynthates isotopically more depleted in ^{13}C than the original carbon source. A greater abundance of CO_2 increases Rubisco-based isotopic discrimination, resulting in an even lower $^{13}C/^{12}C$ ratio ($\delta^{13}C$) in photoautotroph biomass (Farquhar et al., 1989; Farquhar et al., 1982; Francois et al., 1993; Popp et al., 1989). When this phototrophic biomass is preserved in the geologic record, the $\delta^{13}C$ of sedimentary organic matter can be used to reconstruct PCO_2 (Hayes et al., 1999). The largely mixed contributions and diagenetic processes on bulk organic matter can, however, mask this signal (Hayes, 1993; Hayes et al., 1999). Thus, isotope analysis of specific biomarker lipids is preferred in order to better define the source of the $\delta^{13}C$ signal (Jasper and Hayes, 1990; Pagani, 2002).

The most studied biomarkers for calculating ε_p are alkenones, i.e. long-chain unsaturated methyl and ethyl ketones produced by select Haptophytes (Volkman et al., 1980; de Leeuw et al., 1980). The stable carbon isotopic fractionation of alkenones has been studied using cultures and mesocosms with controlled environments (Laws et al., 1995;Benthien et al., 2007), but conditions do not always mimic natural environments and the natural variation in carbonate chemistry that occurs on a daily to seasonal time scales. Furthermore, these experiments are also time-consuming given that they must have delicately balanced water chemistry including $CO_{2[aq]}$ concentrations, pH, and alkalinity, as well as nutrients such as nitrate and phosphate (Popp et al., 1998; Laws et al., 1995; Bidigare et al., 1997), along with the additional challenge of maintaining a constant $\delta^{13}C$ of the $CO_{2[aq]}$ while photoautotrophs continually enrich the growth water as they fix CO_2. Water column studies (Bidigare et al., 1997) and surface sediments (Pagani, 2002) have been applied but rarely reach elevated PCO_2 levels such as those encountered in the past.

Here we use an alternative approach by analyzing algal lipids near natural CO_2 seep systems. In tectonically active zones, volcanically induced seeps consistently bubble high PCO_2 concentrations into the surrounding water, substantially increasing the local CO_2 concentrations in the water and providing an environment referent to past and future high-CO_2 worlds. CO_2

seeps were previously overlooked for biological studies due to the typically high sulfide (H_2S) concentrations associated with volcanic degassing that make these environments largely uninhabitable (Dando et al., 1999). However, certain CO_2 seep systems have been found to have low H_2S concentrations making them suitable for ocean acidification experiments (Hall-Spencer et al., 2008), prompting further research in e.g. the Mediterranean (Boatta et al., 2013), in Japan (Agostini et al., 2015), Papua-New-Guinea (Fabricius et al., 2011), and New Zealand (Brinkman and Smith, 2015). These sites may provide an ideal testing ground for the impact of isotopic fractionation on algal lipids where environmental conditions are at naturally balanced levels with the exception of the large gradient of CO_2 concentrations.

In our study, we explore the relationship between ε_p and $CO_{2[aq]}$ across several pre-established sites with different (temporally consistent) levels of pCO_2 at the warm-temperate CO_2 seep at Mikama Bay off the shore of Shikine Island, Japan. We test this relationship using general algal biomarkers, i.e. compounds derived from a multitude of species and which have rarely been used for ε_p-based pCO_2 reconstructions despite their potential utility (Witkowski et al., 2018; Popp et al., 1989; Freeman and Hayes, 1992).

2 Materials and Methods

2.1 Sample site

The site is briefly described here. For further details, we refer to Agostini et al. (2018). Mikama Bay is located on the south-southwest corner of Shikine Island off the Izu Peninsula, Japan bay (34.320865 N, 139.204868 E) with several venting locations in the north of the bay (Fig. 1)~~(34.320865 N, 139.204868 E)~~. The gas emitted from the seep contains 98% CO_2 and the bay has a spatially and temporally constant total alkalinity averaging at 2265 ± 10 μmol kg^{-1}. Samples were collected from three preestablished pCO_2 sites (Agostini et al., 2015), "Control pCO_2" site in an adjacent bay outside the influence of the CO_2 seep (pCO_2 309 ± 46µatm), a "Mid pCO_2" site (pCO_2 ca. 460 ± 40 μatm), and a "High pCO_2" site (pCO_2 769 ± 225) (Fig. 1). pCO_2 estimates are based on the carbonate chemistry parameters (pH_{NBS}, temperature, salinity, and total alkalinity) of water in the bay and calculated using the program CO2sys (Agostini et al., 2018; Harvey et al., 2018). Temperature (annual range ca. 14 to 28ºC) and salinity (ca. 34‰) are relatively homogenous throughout the bay and do not differ between the elevated pCO_2 sites and control pCO_2 sites (Agostini et al. 2018). Currents and wind were measured in October 2014 and April 2015 (Agostini et al., 2015). October 2014 measurements showed moderate turbulent winds (ranging from 0.6-11.5 m s^{-1}, average 4.5 m s^{-1}) associated with current velocities (ranging from 0 to 1.6 knots, average 0.4 knots) at 5 m in the surface water, whereas April 2015 measurements showed moderate north-northeast winds (1.5-8.6 m s^{-1}, average 5.1 m s^{-1}) associated with low current velocities (0-0.2 knots, average 0.04 knots). Monthly surveys in the bays over the past five years show that these sites have similar annual mean values for temperature, salinity, and currents. Weather station data shows that the severity of seasonal extreme weather event (e.g. typhoons) varies on an annual basis (Japan Meteorological Agency, https://www.jma.go.jp/en/typh/).

2.2 Materials

Samples were collected in June and September of 2016 (indicated in Fig. 1). All samples were collected in at least triplicate for each site ("Control PCO_2", "Mid PCO_2" and "High PCO_2" site). Additional control sites (at ca. 1.8 km and 2.4 km away from the CO_2 seep) around the island were taken to ensure the fidelity of the Control site closest to the seep. June sampling included surface waters for dissolved inorganic carbon (DIC) measurements, surface sediments, and benthic diatoms attached to surface sediment through extracellular polymeric substance production. In September, macroalgae~~,~~ ~~suspended particulate matter (SPM),~~ and plankton tows were collected, in addition to surfaced water DIC and surface sediments, taken in triplicate at each site on four separate days.

Water for the $\delta^{13}C$ of DIC analysis was collected by overfilling glass vials with sea surface water and adding mercury chloride (0.5%) before closing with a septa cap and sealing with electrical tape. Surface sediments were collected by divers using geochemical sample bags. Macroalgae and benthic diatoms were scraped off submerged rocks at each respective site. ~~SPM was sampled by collecting sea surface water in three 23 L Nalgene tanks (20 L filtered from each tank) and taken back to the lab where 60 L per site per day were filtered over a single 0.7 μm GFF.~~ A 25 μm mesh plankton net ("small plankton net", Rigo, Saitama, Japan) was towed 50 m three times per site and filtered using a portable hand aspirator on the boat over a single 0.7 μm ~~47 mm muffled~~ GFF (combusted prior to sampling for 4 h at 450°C). All samples were immediately frozen; once back in the lab, these were freeze-dried and kept in a refrigerator.

2.3 Methods

Each seawater sample was measured for the $\delta^{13}C$ of DIC in duplicate on a Thermo Scientific Gas Bench II coupled to a Thermo Scientific Delta V mass spectrometer. Prior to running samples, 12 ml vials were prepared with 100 μL of 85% H_3PO_4 and flushed with He. 500 μL of seawater was ~~then~~ added and left to react for at least 1 h prior to measuring the headspace. Standards were run at the start, end, and every six runs of a sequence. Standards were prepared with 0.3 mg of Na_2CO_3 and 0.4 mg of Ca_2CO_3 (all calibrated against NBS-19) ~~which were then~~ flushed with He, injected with 500 μL of 85% H_3PO_4, and reacted for 1 h. The headspace was ~~then~~ measured. ~~A~~and average values and standard deviation errors reported are based on six measurements for June (three at the High PCO_2 site and three at the Control) and thirty-six measurements for September (three each at the High PCO_2 site, Mid PCO_2 site, and Control collected on four separate days).

Freeze-dried sediments, benthic diatoms, and macroalgae were homogenized using mortar and pestle and extracted using a Dionex 250 accelerated solvent extractor at 100°C, 7.6x106 Pa using dichloromethane (DCM): MeOH (9:1 v/v). GFFs containing plankton net material ~~or SPM~~ were cut into 1 mm x 1 mm squares and extracted using ultrasonication (5x) with 2 ml dichloromethane (DCM): MeOH (9:1 v/v) ~~five times~~. All total lipid extracts (TLEs) were hydrolyzed by refluxing the TLE with 1N of KOH in MeOH for one hour and neutralized to pH 5 using 2 N of HCl in MeOH. Bi-distilled water (2 ml) and DCM (2 ml) were added (5x) to the hydrolyzed centrifuge tubes and the organic matter in the DCM layers ~~with~~ were pooled ~~organic matter was removed~~ and dried over Na_2SO_4. ~~After drying t~~The resulting ~~base~~ hydrolyzed TLE~~s, samples~~ were

eluted over an alumina packed column and separated into apolar (hexane: DCM, 9:1 v/v), ketone (DCM), and polar (DCM: MeOH, 1:1 v/v) fractions. Polar fractions were ~~then~~ silylated using pyridine: N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (1:1 v/v) and heated at 60°C for 1 h prior to ~~running~~ analyses on the gas chromatography-flame ionization detector (GC-FID), gas chromatography-mass spectrometry (GC-MS), and gas chromatography isotope-ratio mass spectrometry (GC-IRMS).

Silylated polar fractions were analyzed by GC-FID ~~to check the baseline quality of the sample and to determine quantities of compounds~~for quantification. Based on the quantities, fractions were diluted with ethyl acetate and ca. 1 ug of polar fraction was injected on-column for GC-MS to identify compounds and for GC-IRMS to measure the isotopic composition of specific compounds. Each instrument is equipped with the same CP-Sil 5 column (25 m x 0.32 mm; df 0.12 µm) and He is used as carrier gas. GC oven was programmed from ~~FID, GC-MS, and GC-IRMS had starting oven temperatures of~~ 70ºC to 130ºC ~~ramped~~ at 20ºC/min ~~to 130ºC~~ and then to 320ºC ~~ramped~~ at 4ºC/min ~~to 320ºC~~ which was held for 10 min. All three instruments use the same in-house mixture of *n*-alkanes and fatty acids to check chromatography performance at the start of each day (GC-standard). For compound specific stable carbon isotope analysis using GC-IRMS, additional standards with known isotopic values (-32.7 and -27.0‰) of per deuterated (99.1%) *n*-alkanes (C_{20} and C_{24}, respectively), were co-injected with the GC-standard. Samples were also co-injected with the ~~C_{20} and C_{24}~~same GC-IRMS standards to monitor instrument performance. Every day, the Isolink II combustion reactor of the GC-IRMS was oxidized for at least 10 min, backflushed with He for 10 min, and purged for 5 min; a shorter version of this sequence is conducted in post-sample seed oxidation which includes 2 min oxidation, 2 min He backflush, and 2 min purge conditioning line. Longer oxidations were run weekly. Each derivatized compound was corrected for the $\delta^{13}C$ of the BSTFA used in silylation (-32.2‰).

3 Results

Samples from the different matrices were collected at several Control PCO_2 sites (309 ± 46, at a "Mid PCO_2" site (ca. 100 m from the venting area; 460 ± 40 μatm), and near the venting area ("High PCO_2" site; 769 ± 225 μatm) during June and September 2016 (Fig. 1), which included June-collected surface waters (for DIC), surface sediments, and benthic diatoms, and September-collected surface waters (for DIC ~~and SPM~~), surface sediments, plankton net tows, and macroalgae. ~~With the exception of the SPM from surface waters, all samples yielded enough material for isotope studies, and therefore phytoplankton filters from surface waters were not included in this study.~~

The $\delta^{13}C$ of DIC demonstrated minimal change over the gradient of CO_2 and minimal change between the two seasons (Fig. S1). The June $\delta^{13}C$ of DIC was 0.2 ± 0.2 ‰ (± SD; N=3) at the Control site and 0.5 ± 0.04 ‰ (N=3) at the High PCO_2 site. The September $\delta^{13}C$ of DIC was -0.4 ± 0.2 ‰ (N=8) at the Control site, -0.1 ± 0.1 ‰ (N=8) at the Mid PCO_2 site, and 0.2 ± 0.4 ‰ (N=8) at the High PCO_2 site.

The polar fractions of the extracts of the surface sediments, plankton, macroalgae, and benthic diatoms showed a similar suite of compounds, observed across all sites and during both seasons. The most prominent compounds were loliolide,

phytol, C_{14}-C_{16} alkanols, and sterols such as cholesta-5,22E-dien-3β-ol, cholesterol, 23-methylcholesta-5,22dienol, campesterol, stigmasterol, and β-sitosterol (e.g. Fig. 2). Terrestrial biomarkers, such as long chain alcohols and triterpenoids were not ~~-~~detected. Loliolide, phytol, and cholesterol were targeted for stable carbon isotope analysis as the most abundant general algal biomarkers and with relatively good separation in the GC. The biological sources of these compounds will be discussed in Section 4.1.

Among the sample matrices, the $\delta^{13}C$ of loliolide ranges from -19.8 to -22.0 ‰ at the Control sites, from -20.5 to -22.9 ‰ at the Mid PCO_2 site, and from -23.1 to -29.0 ‰ at the High PCO_2 site (Fig. 3A; Table S1). The $\delta^{13}C$ of loliolide from June surface sediments shows the strongest change from the Control site to the High PCO_2 site (-21.2 to -29.0 ‰), followed by the $\delta^{13}C$ of loliolide from September macroalgae (-21.3 to -25.7 ‰). A lesser $\delta^{13}C$ shift is observed in the September surface sediment-derived loliolide (-19.8 to -23.1 ‰). The $\delta^{13}C$ of the benthic diatom-derived loliolide (-20.2 to 23.6 ‰) and the plankton tow-derived loliolide show the smallest shifts from the Control to High PCO_2 site (-22.0 to -23.6 ‰).

Similar to the results of the $\delta^{13}C$ of loliolide, the $\delta^{13}C$ of phytol also consistently shows higher $\delta^{13}C$ values in the Control sites and lower $\delta^{13}C$ values in the elevated PCO_2 sites among all samples types collected in both seasons (Fig. 3B; Table S1). For the whole sample set, the $\delta^{13}C$ of phytol ranges from -18.9 to -22.6 ‰ at the Control site, from -19.4 to -22.4 ‰ at the Mid PCO_2 site, and from -22.6 to -27.8 ‰ at the High PCO_2 site (Fig 3B), similar ranges as observed for loliolide. A similar shift in $\delta^{13}C$ values of phytol is observed with increasing PCO_2 in the June surface sediments (-22.6 to -27.8 ‰), the June benthic diatoms (-18.9 to -24.4 ‰), and the September macroalgae (-21.5 to -26.9 ‰). Smaller changes in the $\delta^{13}C$ of phytol are observed for September plankton (-21.7 to -24.4 ‰) and September sediment (-20.5 to -22.6 ‰).

The $\delta^{13}C$ of cholesterol likewise shows a similar trend to the other two biomarkers but with a smaller shift in the $\delta^{13}C$ values from the Control PCO_2 sites to the elevated PCO_2 sites. Among the different sample matrices, the $\delta^{13}C$ of cholesterol ranges from -21.2 ‰ to -25.1 ‰ at the Control site, -22.1 to -23.4 ‰ at the Mid PCO_2 site and -23.1 to -27.4 ‰ at the High PCO_2 site (Fig. 3C; Table S1). The strongest change in the $\delta^{13}C$ of cholesterol with increase PCO_2 occurs in the June surface sediments from -22.6 ‰ in the Control to -27.8 ‰ at the High PCO_2 site. The June benthic diatoms also have a large isotopic shift in the $\delta^{13}C$ of cholesterol (-21.2 to -25.8 ‰), as does the September macroalgae (-22.7 to -25.8 ‰). The September surface sediments (-22.2 to -23.1 ‰) and plankton tow-derived cholesterol (-25.1 to -26.7 ‰), however, have a smaller shift from the control to the elevated PCO_2 sites.

4 Discussion

4.1 The $\delta^{13}C$ differences in biomarkers among matrices and seasons

All three biomarkers, phytol, loliolide and cholesterol, show a negative shift in $\delta^{13}C$ values with increasing PCO_2 in each matrix and each season (Fig. 3), agreeing with the theory that higher PCO_2 conditions result in ~~lighter~~ lower $\delta^{13}C$ values in

biomass (Farquhar et al., 1982). However, despite all having algal sources, the absolute isotope values vary for 1) each compound, 2) each matrix, and 3) both seasons, which we will now discuss.

First, the absolute values of $\delta^{13}C$ values vary among the three compounds. This may be expected given the different biosynthetic pathways leading to formation of each compound (Schouten et al., 1998), as well as the different contributors to each compound. Loliolide, considered a diatom biomarker in paleoreconstructions (e.g. Castañeda et al., 2009), is a diagenetic product of fucoxanthin (Repeta, 1989; Klok et al., 1984), a xanthophyll which contributes to approximately 10% of all carotenoids found in nature (Liaaen-Jensen, 1978). Phytol, considered a photoautotroph biomarker in paleoreconstructions (Hayes et al., 1990), is the side-chain of the vital and omnipresent pigment chlorophyll *a* that directly transfers sunlight energy into the photosynthetic pathway in nearly all photosynthetic organisms. Sterols, considered a general eukaryotic biomarker in paleoreconstructions, are the eukaryotic tetracyclic triterpenoid lipids used for critical regulatory roles of cellular functions e.g. maintaining membrane fluidity (Nes et al., 1993). Although sterols are virtually restricted to eukaryotes, some exceptions have been found in bacteria (Wei et al., 2016). Here we only examine cholesterol, which is universally absent in prokaryotes and composes of up to 20-40% of eukaryotic plasma membranes (Mouritsen and Zuckermann, 2004). Phytol and cholesterol may also have terrestrial sources given that they are derived from all photoautotrophs and all eukaryotes, respectively. However, these samples were taken off the coast of a small island in open ocean and the absence of characteristic terrestrial biomarkers indicates that terrestrial contributions can be considered to be minimal. The close resemblance of the isotopic composition among all three compounds, including the primarily diatom-limited compound loliolide, suggests that these compounds share relatively similar source organisms. Cholesterol shows a lessened isotopic shift than the other two compounds from the ambient to elevated PCO_2 sites. Although we cannot fully exclude that this is due to terrestrial input, it is more likely due to the mobile eukaryotic zooplankton in the water column which also contribute to the cholesterol signal.

Within the same biomarker and same season, some differences among matrices were observed. This difference may be due to the mobility of the matrix, as well as the algal assemblages. The plankton tow which captured free-floating surface water algae from that specific growth season is more readily transported by wind than the surface sediment, which likely reflects the culmination of multiple growth seasons throughout the water column. This is seen, for example, in the $\delta^{13}C$ of cholesterol collected in September from the same Control site where surface sediments are -22.2 ‰ and plankton tows are -25.1 ‰, where the latter has possibly been transported from sites with elevated PCO_2 levels. Similar differences among matrices are also observed in phytol and loliolide. The hypothesis of transportation affecting the isotopic signal in certain matrices is supported by the results from the macroalgae. The macroalgae, in contrast to the algae collected by plankton tows, were unaffected by transportation due to being fixed to the nearby rocks at each site. Thus, the isotopic composition of compounds of the macroalgae was similar to that of the long-accumulated surface sediments, e.g. -22.7 ‰ for the $\delta^{13}C$ of cholesterol at the September Control site.

Finally, there is a difference in the $\delta^{13}C$ values for biomarkers between seasons. The June-collected surface sediments and algae yielded a larger difference in $\delta^{13}C$ values along the CO_2 gradient than the September-collected surface sediments and

algae. This seasonal difference may be due to extreme weather conditions experienced between the two sampling campaigns. Although typhoons are common in this region, in the weeks preceding the fieldwork in September, Shikine Island experienced an unusually high quantity of storms. The storms were also of unusual strength for this region of the Pacific, including Typhoons Mindulle and Kompasu, the severe tropic storms Omais and Chanthu, and the long-lived, erratic Lionrock typhoon. This atypical abundance and severity of storms observably ripped corals out of the rocks around Shikine Island and thus likely resuspended and transported some sediment around the bay. This would explain the reduced $\delta^{13}C$ difference between the Control and High PCO_2 site in the surface sediments collected in September, as well as the readily transportable algae collected by the plankton tow, and would explain why the rock-affixed macroalgae, also collected in September, maintained a strong $\delta^{13}C$ change across the transect.

4.2 The ε_p among general algal biomarkers

To further validate the impact of PCO_2, we calculated the isotopic fractionation of algal biomass based on the $\delta^{13}C$ of the three biomarkers. Here we focus on surface sediments as they are a close analogue to the geological sediment records. Although the macroalgae and benthic diatoms also show strong isotopic fractionation, they represent a limited number of species and a single growth season. Furthermore, we calculated the ε_p from the June-collected surface sediments, which appear to be the least affected by typhoon activity and represent fractionation over multiple seasons.

To calculate ε_p in the June-collected surface sediments, we correct the $\delta^{13}C$ of the organic matter (δ_p) for the $\delta^{13}C$ of the inorganic carbon source for the producers of these compounds (δ_d) in Eq. (1):

$$\varepsilon_p = 1000 \cdot [(\delta_d+1000) / (\delta_p+1000) - 1] , \quad (1)$$

δ_p is calculated by correcting the $\delta^{13}C$ for each individual biomarker for the offset with photosynthetic biomass caused by isotopic fractionation during biosynthesis. The isotopic offset between phytol and biomass is 3.5 ± 1.3 ‰ based on the average of twenty-three species compiled in Witkowski et al. (2018) and the isotopic offset between sterols and biomass is 4.5 ± 3.0 ‰ based on the average of eight algal species (Schouten et al., 1998). The isotopic offset for loliolide from biomass, however, has not been determinedstudied. Because isoprenoids are formed from the same biosynthetic pathway, we here average the offset of the other two isoprenoids here (4.0 ‰) to estimate a value for the difference between loliolide and biomass.

δ_d is calculated by correcting the measured $\delta^{13}C$ of DIC for temperature (Mook, 1974) and pH (Madigan et al., 1989), which considers the relative contribution of different inorganic carbon species to the measured DIC. Based on the equations of Mook et al. (1974), we correct for the temperature-dependent carbon isotopic fractionation of dissolved CO_2 with respect to HCO_3^- using the annual mean sea surface temperature for Shikine Island of 20.4ºC (Agostini et al., 2018). Based on the equations of Madigan et al. (1989), we corrected for the $\delta^{13}C$ of HCO_3^- and $\delta^{13}C$ of $CO_{2[aq]}$ mass balance calculation that accounts for the relative abundance of these inorganic carbon species based on pH (Lewis and Wallace, 1998) at the High PCO_2 site (7.81 pH_T) and Mid PCO_2 site (7.99 pH_T) relative to the ambient Control (8.14 pH_T). The corrected δ_d values yield -10.1 ‰ at the Control site, -10.0 ‰ at the Mid PCO_2 site, and -9.5 ‰ at the High PCO_2 site (Table S2).

ε_p values consistently yield much higher values at the elevated PCO_2 sites than the ambient Control sites for~~in~~ all three biomarkers, which share similar trends and absolute values (Fig. 4; Table S3). ε_p derived from loliolide averages 7.2 ± 1.6 ‰ at the Control, 9.2 ± 1.6 ‰ at the Mid PCO_2 site, and 15.9 ± 1.6 ‰ at the High PCO_2 site, ε_p derived from phytol at 8.6 ± 0.4 ‰, 8.6 ± 0.9 ‰, and 14.9 ± 1.0 ‰, respectively, and ε_p derived from cholesterol at 7.6 ± 3.0 ‰, 9.2 ± 3.1 ‰, to 13.7 ± 3.1 ‰, respectively, where errors represent the standard deviation of the triplicate samples taken at each site. These results show that CO_2 has a profound impact on ε_p as it is the only variable with a large gradient in the bay. Given that maximum fractionation for algae species is ca. 25 to 28 ‰ in laboratory cultures (Goericke and Fry, 1994), the CO_2 seep values suggests strong, but ~~not close to maximum, fractionation of the local algae.~~ does not approach maximum fractionation (ε_f) at the high CO_2 site. This may be due to presence of carbon concentrating mechanism in phytoplankton which utilize ^{13}C-enriched bicarbonate or possible due to the presence of Rubisco types with different ε_f values than previously assumed (Thomas et al., 2018).

4.3 PCO_2 reconstructed from general algal biomarkers

We estimate PCO_2 from the ε_p values, a relationship first derived for higher plants (Farquhar et al., 1989; Farquhar et al., 1982) and later adapted for algae (Jasper et al., 1994; Rau et al., 1996) in Eq. (2):

$$PCO_2 = [\, b / (\varepsilon_f - \varepsilon_p) \,] / K_0 \quad , \qquad (2)$$

where ε_f reflects the maximum Rubisco-based isotopic fractionation, b reflects species carbon demand per supply such as growth rate and cell-size (Jasper et al., 1994), and K_0 reflects a constant to convert $CO_{2[aq]}$ to PCO_2 based on temperature and salinity (Weiss, 1974). ε_f for algal species range from 25 to 28 ‰ in laboratory cultures (Goericke and Fry, 1994); we use an average 26.5 ‰ with an uncertainty of 1.5 ‰ uniformly distributed for these general algal biomarkers (Witkowski et al., 2018). The b value is difficult to estimate as it is a catchall for factors other than PCO_2 that affect fractionation and is particularly difficult to estimate for general algal biomarkers because they are derived from a multitude of species. Previous studies using phytol's diagenetic product phytane as a PCO_2 proxy (Bice et al., 2006; Sinninghe Damsté et al., 2008; van Bentum et al., 2012) have used a mean value of 170 ‰ kg μM^{-1}, similar to the mean of alkenone-producers. This is supported by a compilation of the $\delta^{13}C$ values of modern surface sediment organic matter mean average of 168 ± 43 ‰ kg μM^{-1} (Witkowski et al., 2018) and a single study on phytol in the equatorial Pacific Ocean (Bidigare et al., 1997). We apply this average, rounded to 170 ± 50 ‰ kg μM^{-1} to all three general algal biomarkers.

The resulting reconstructed PCO_2 estimations show the expected values in the Control sites and much higher values in the elevated CO_2 sites among all three biomarkers (Fig. 5; Table S3). Loliolide shows the biggest shift, from 239 +50/-49 μatm at the Control, 266 +57/-54 μatm at Mid PCO_2 site, and 437 +113/96 μatm at the High PCO_2 site. Phytol has similar but a slightly smaller shift in PCO_2 estimates to loliolide, with estimations of 264 +55/-54 μatm, 291 +56/-53 μatm, and 444 +98/-87 μatm at the Control, Mid PCO_2 site, and High PCO_2 site, respectively. Cholesterol shifts similarly to the other two biomarkers with 244 +67/-54 μatm, 266 +77/-61 μatm, and 358 +136/-90 μatm, respectively. These reconstructed values closely match each other and trend in the same direction as the actual values.

The reconstructed PCO_2 values derived from the $\delta^{13}C$ of general algal biomarkers closely match the actual measured PCO_2 values of the Control (Fig. 5), i.e. 309 ± 46 μatm (Agostini et al., 2018; Harvey et al., 2018), when considering the uncertainty in the reconstructed estimations. However, the proxies underestimate the absolute values measured at the elevated PCO_2 sites (Fig. 5; Table S3), i.e. 460 ± 40 μatm at the Mid PCO_2 site and 769 ± 225 μatm at the High PCO_2 site (Agostini et al., 2018; Harvey et al., 2018). There are several possible explanations to why there is an underestimation. As discussed above, carbonate concentration mechanisms may be operating in a large number of phytoplankton, such that they become relatively enriched in ^{13}C and thus lead to lower reconstructed PCO_2 values (Stoll et al., 2019; Badger et al., 2019). There is also a large uncertainty in the *b* value applied, which may be much lower than the value assumed here. However, if so, then PCO_2 values reconstructed for past times may be much higher, leading to considerable discrepancies with other PCO_2 proxies (c.f. Witkowski et al., 2018). A simple explanation for t~~T~~his underestimation may be ~~caused by~~ some site limitations. The high variability of PCO_2 at these sites could have impacted the reconstructed values, as these algae could have been exposed to much different, and perhaps lower, levels than those observed during the times that PCO_2 values were measured. Furthermore, there is a strong possibility of allochthonous marine input of sediment at the Mid and High PCO_2 site, i.e. input from sediment outside of the bay area. This allochthonous input seems likely given the intense weather conditions that occur annually in this small bay in which lateral transport of sediment could bring algal material grown in ambient PCO_2 conditions into the bay and dampen the overall PCO_2 signal picked up in the biomarkers. Future research conducted at another CO_2 seep settings with different weather and current conditions could illuminate this.

5 Conclusion

We analyzed the $\delta^{13}C$ of general algal biomarkers in surface sediments, plankton, benthic diatoms, and macroalgae collected in a transect from a CO_2 vent during two seasons. The strong $\delta^{13}C$ change between the Control and elevated PCO_2 sites suggest that the increased CO_2 concentrations in the seawater does indeed influence fractionation of photoautotrophic biomass and validates previous PCO_2 reconstructions which have considered utilizing general algal biomarkers for this purpose. Reconstructions correctly estimate control values, though reconstructions at the elevated PCO_2 sites show underestimations of the actual PCO_2, ~~likely~~ possibly due to the allochthonous input from nearby marine sediments deposited under normal PCO_2 levels caused by the intense annual typhoon activity in this region. Our results show that CO_2 seeps may offer testing grounds for exploring new PCO_2 proxies under natural conditions at high PCO_2 levels such as those encountered in the geological past.

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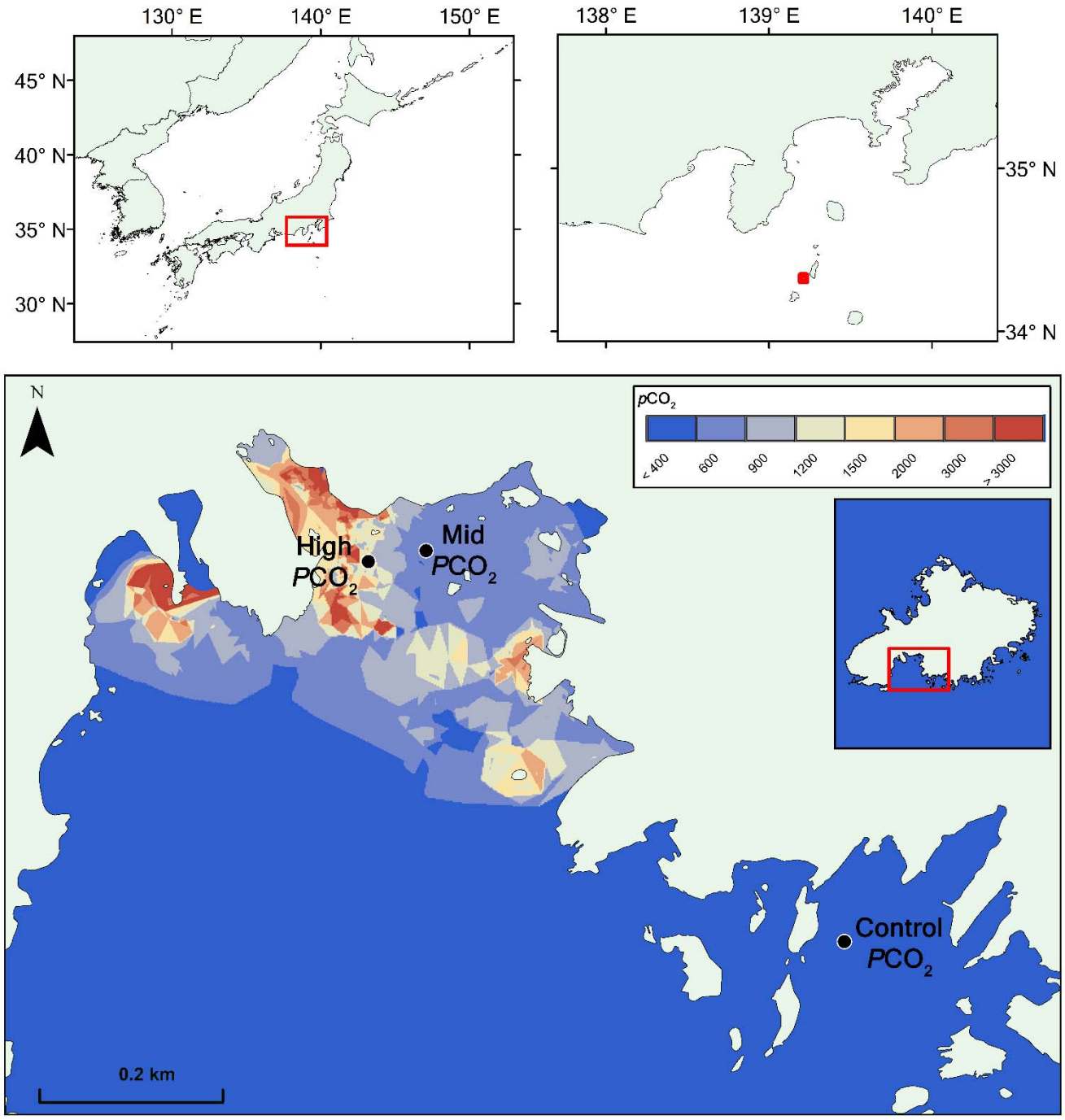

Figure 1: Map of PCO_2 in the study region at Shikine Island (Japan). Top panel shows geographical context. Lower panel shows the bay on Shikine Island, with ~~S~~spatial variability in PCO_2 (Agostini et al., 2018), computed using the nearest neighbor algorithm in ArcGIS 10.2 software (http://www.esri.com/software/arcgis/).

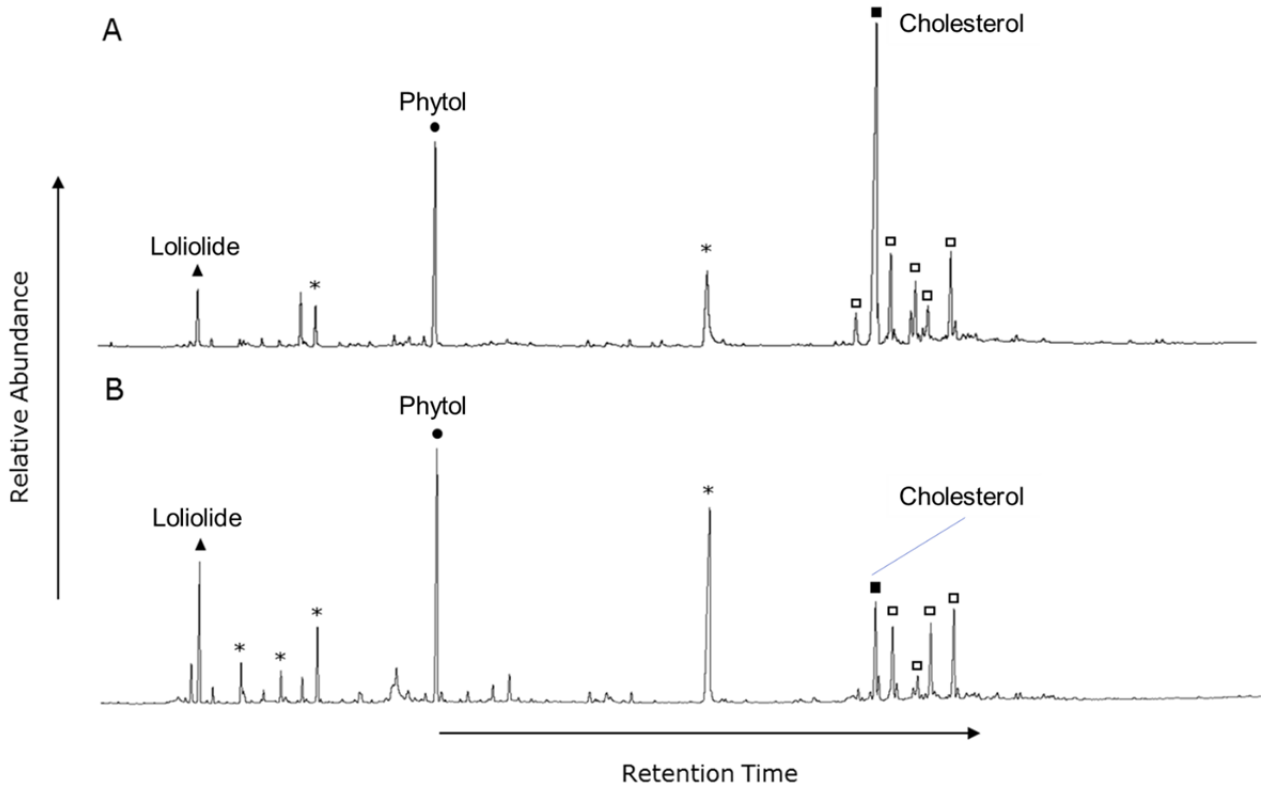

Figure 2: ~~Chromatogram~~ GC-FID trace of silylated polar fraction. June sediment collected at the A) Control site and B) CO_2 vent, showing saturated fatty alcohols (asterisk) and sterols (square), and the representative compounds found among all sample matrices, seasons, and CO_2 concentrations: loliolide, phytol, and cholesterol.

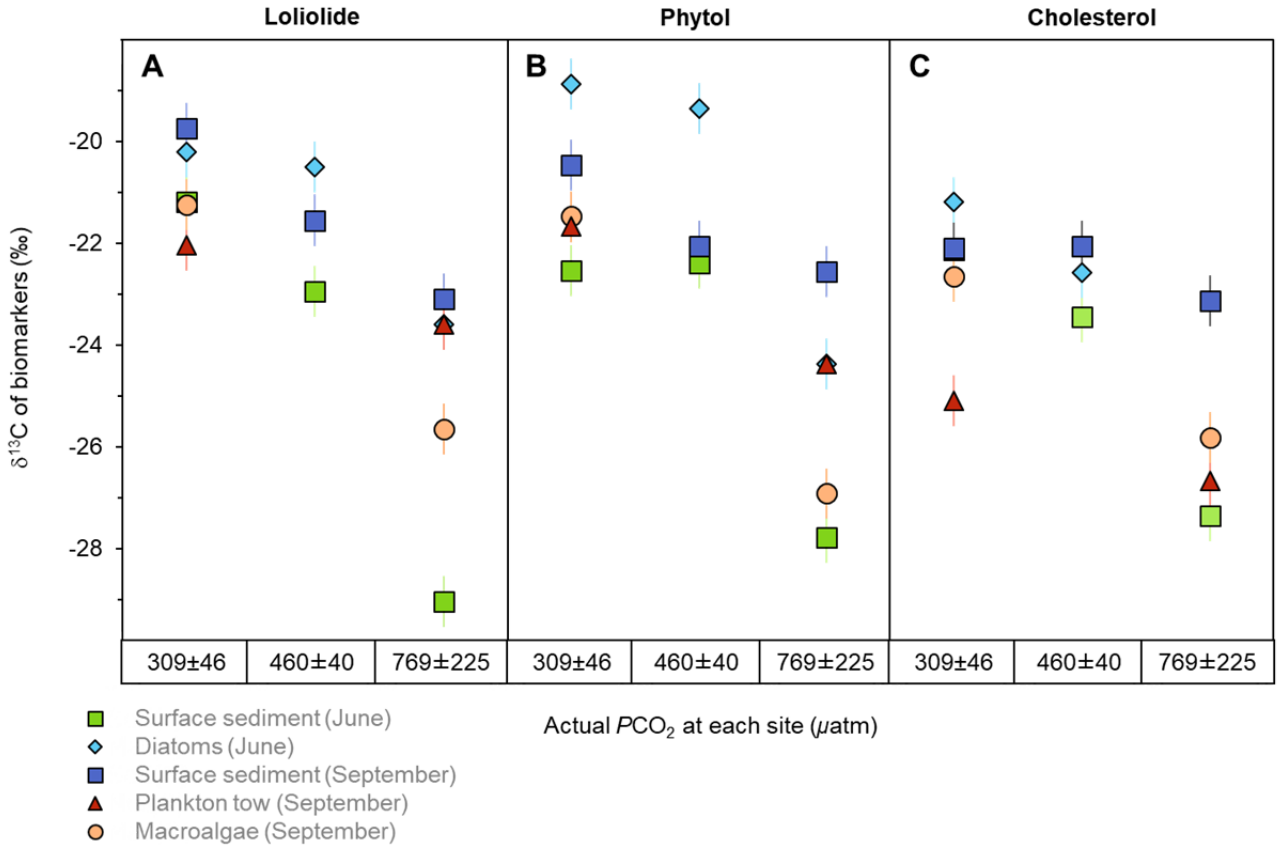

Figure 3: The $\delta^{13}C$ of general algal biomarkers in sediments. A) Loliolide, B) phytol, and C) cholesterol from the Control, Mid, and High PCO_2 sites during June and September from different sample matrices, including surface sediment (square), benthic diatoms (diamond), plankton tow (triangle), and macroalgae (circle).

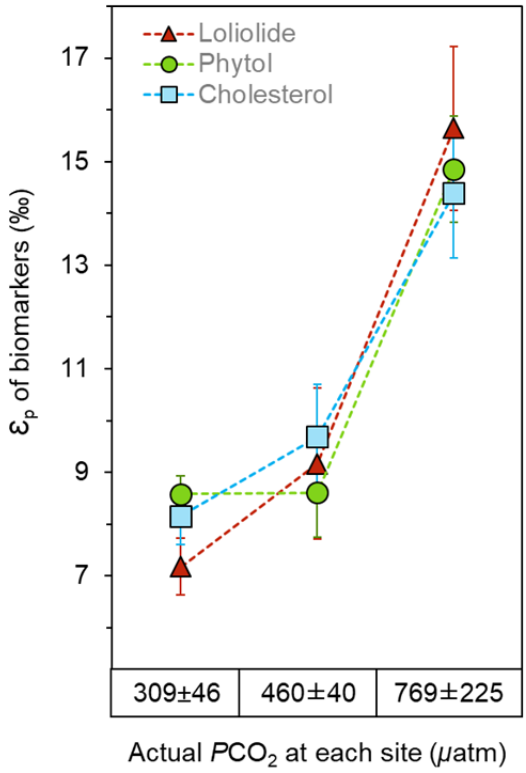

Figure 4: The ε_p of general algal biomarkers in sediments. Loliolide (triangle), phytol (circle), and cholesterol (square) from the Control, Mid and High PCO_2 sites during June sediment collection.

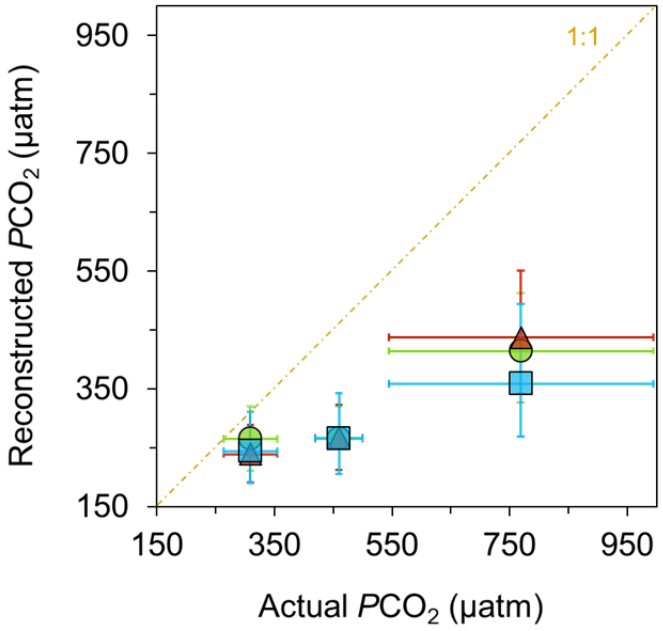

Figure 5: Reconstructed PCO_2 from general algal biomarkers. PCO_2 reconstructed from the $\delta^{13}C$ of loliolide (triangle), phytol (circle), and cholesterol (square) in June-collected sediments versus the actual PCO_2 measured at each location (Agostini et al., 2018; Harvey et al. 2018).

5

Supplementary material

Table S1. The $\delta^{13}C$ of biomarkers from different matrices. The $\delta^{13}C$ of general biomarkers loliolide, phytol, and cholesterol measured during two seasons from sea surface sediments, diatom mats, plankton net tows, and macroalgae.

Table S2. The $\delta^{13}C$ of CO_2. All parameters used to calculate the $\delta^{13}C$ of CO_2 (represented in the equations as δ_d), including corrections for sea surface temperature and pH.

Table S3. The $\delta^{13}C$ of algal biomarkers and all parameters used to estimate pCO_2. All parameters used to calculate pCO_2 from the $\delta^{13}C$ of general algal biomarkers: loliolide, phytol, and cholesterol.