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Interactive comment on "Do marine benthos breathe what they eat?" *by* Xiaoguang Ouyang et al.

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This paper aims to determine if "you breathe what you eat", specifically, whether the d13C value of carbon dioxide respired by benthic fauna (crabs and gastropods) from a mangrove forest reflects the d13C of the dietary source. The authors used four separate experiments to determine how d13C of respired carbon is affected by 1) taxon (crabs vs gastropods), 2) feeding mode (detritivores vs deposit-feeders), and 3) food source (MPB vs senescent mangrove leaves, and 4) how the quantity of respired CO2 is affected by animal size. The flux and d13C of respired CO2 was determined through incubation of fauna within sealed chambers, with CO2 sampled periodically via syringe, and analysed using a Picarro CRDS. Although there are some very clear differences and trends in the data collected, I un- fortunately remain unconvinced that this study

represents a useful advance in isotope techniques and I remain uncertain of the conclusions. The manuscript should be revised to improve readability through correction of grammatical errors. However, it is most important that the manuscript be revised to more clearly and thoroughly outline the basis for the study and the hypothesis of the various experiments, and to clearly explain how the outcomes of the study advance the field and can be practically applied elsewhere.

Response: We will revise the ms to correct some grammatical errors, such as replacing δ13C of deposit feeders respired CO2 with δ13C-CO2 respiration for deposit feeders throughout the ms. We will clearly present the justification, aim and hypothesis of each experiment. We will clearly show the advantage of our method in comparison with classical tissue isotope studies, and specify the alternative for CRDS so that the concept 'You Breathe What You Eat' can be applied elsewhere. Specifically, we have clarified how we will improve the ms in the following responses to the reviewer's concerns and specific comments.

My main concerns are as follows: 1) As per the comment above, the justifications, aims, and hypotheses of each of the experiments should be clearly outlined.

Response: We aim to put forward the novel notion 'You Breathe What You Eat', which adds to the well-known paradigm of 'You Are What You Eat' in food web studies. Specifically, whether the ïĄď13C-CO2 respiration for marine benthos reflects their taxonomic background, size and dietary source. Our specific hypotheses include (1) ïĄď13C-CO2 respiration is dependent on the taxonomic background of benthic consumers; (2) ïĄď13C-CO2 respiration reflects their feeding habit; (3) ïĄď13C-CO2 respiration and CO2 production is influenced by the feeding regime; and (4) benthos CO2 production is dependent on their size. We shall rearrange the Results section to make it correspond to each experiment described in the Materials and Methods section and hypotheses outlined in the Introduction section, to improve correspondence between results of each experiment and the relevant hypothesis.

2) Given that d13C values for the leaf and MPB are similar, and 13C fractionation between diet and respired CO2 is apparently âĹij3-10 per mil (Table 1) and presumably somewhat variable, it seems to me that it would be impossible to distinguish leaves and MPB as potential dietary sources on the basis of breath analysis. Even for sources with more distinct d13C values (e.g., C3 and C4 plants) it would be difficult to determine diet, given the large variability in fractionation of d13C-CO2resp vs d13C-diet based on taxa, diet, and feeding mode (based on data in Table 1). It appears that very specific data would need to be collected through targeted experiments before applying the proposed method, which seems to make application of the proposed technique too complex to be practical.

Response: In Fig. 1, we have shown that there is a significant difference between ïĄď13C of mangrove leaf and MPB (t test, P<0.01). Our results show the apparent fractionation between ïĄď13C-CO2 respiration for benthos and their diets (3.1-9.6 ‰ resulting from different benthos taxa and diets. In classical tissue isotope studies, clear fractionation differences are also demonstrated between ïĄď13C of crabs tissues and diets (Bui and Lee 2014) and different benthic consumers (Kristensen et al. 2017). So we think taxa and diet specific isotope fractionation is common for both our approach and the classical tissue isotope approach, and does not hinder its use for identifying the food sources of benthos. The solution is to use diet and taxa specific isotope fractionation to determine benthos diets, as the fractionation value generating from a wide range of consumer-food combinations have been criticized for failing to explain specific trophic paths (Bui and Lee 2014). We shall revise the text to clearly show these points.

3) Assuming it 'works', the advantage of using the proposed technique is unclear. The d13C of respired CO2 (d13C-CO2resp) has been used to determine diets of higher order consumers where it is not feasible to collect tissue samples for 'traditional' isotope analysis using an EA-IRMS. However, this is typically not an issue for intertidal invertebrates, and CRDS is still typically less accessible than EA-IRMS analysis. Where

might the proposed method be of use? Given the rapid shift in d13C-CO2resp it is likely that the CO2 represents very recent diet, and could be used in combination with tissue sampling to determine diet shifts. Could the authors indicate where this might be useful? Furthermore, how might it be possible to distinguish between a shift in recent diet vs a shift to use of stored carbon (e.g. lipids)? How is this distinct from simply analysing tissues with different turnover rates to look at recent changes in diet? It could also be noted that the method does not rely on use of a CRDS, but CO2 samples could also be collected into sealed vials and submitted for analysis via a gasbench/GC-IRMS where a CRDS is unavailable.

Response: We thank the reviewer for suggesting gasbench/GC-IRMS as an alternative to our approach. The concept 'You Breathe What You Eat' does not rely on the use of CRDS but this equipment facilitates the measurements. The usefulness of our method lies in: (1) it can provide information about both the most recently consumed diet and the integrated diet over longer periods while the classic tissue isotope analysis only tracks the integrated diet over time; (2) breath δ13C can be repeatedly measured non-destructively for the same animals and thus can track the changes in its food sources while animals must be sacrificed for the classic tissue isotope analysis which cannot track the change in food sources for the same animal. Our method is useful since some marine crabs remain dormant most of the time with a short active period (e.g. 90 days, Katz 1980). Our experiment has monitored the changes in ïĄď13C-CO2 respiration for benthos and CO2 production when they are fasted or fed on leaf litter/microphytobenthos to reflect their active and dormant status. Some species of aquatic migratory species occupy intertidal habitats during specific seasons of the year. Our experiment has shown the changes of ïĄď13C-CO2 respiration and CO2 production under different feeding regimes to reflect their changes in food during migration. When δ13C-CO2 respiration for benthos is combined with tissue sampling, it might be useful for identifying the shift in recent diet versus using stored energy under starved conditions by analysing tissues with different turnover rates.

4) The title of the paper “Do marine benthos breathe what they eat?” does not reflect the content of the manuscript in its entirety. There is considerable focus on whether marine benthos breathe more when they are larger (CO_2 flux vs size relationships). With regard to this focus, it is not clear how an understanding of CO_2 production vs size is of practical use. The authors mention the potential incorporation of this relationship when determining the contribution of fauna to CO_2 effluxes from mangrove forests, but this would presumably also rely on some understanding of population structure and/or size distribution of benthic taxa. This should be outlined to make clear why this should be of interest.

Response: We shall highlight the significance of the relationships between CO_2 flux and benthos size, combined with population structure and/or size distribution of benthic taxa to determine how much marine fauna contribute to CO_2 effluxes from mangrove forests. The latter data on size distribution and density are, however, beyond the scope of this study.

5) The implications of the large differences in fractionation with taxa, diet, and feeding mode are not fully discussed. Fauna may breathe what they eat, but how can we determine what they eat based on what they breathe?

Response: As explained in our response to the reviewer’s concern (2), we will discuss and clarify the implications of the taxa and diet specific fractionation, and the approach to determine what they eat based on what they breathe.

6) What is the potential impact of confinement and the conditions of the chambers (no sediment, no burrow) on respiration (both in terms of quantity of C respired, and its source (e.g. lipids vs carbohydrates vs proteins))

Response: In experiment (4), we only tried to determine the relationship between benthos respired CO_2 and their sizes. Our previous study has examined the relationship between number of crab burrows and sediment CO_2 flux (Ouyang et al., 2017). These studies and other related studies are useful for partitioning the contribution of marine

benthos and burrows to CO_2 effluxes from mangrove forests. Even in the container, we observed that crabs and gastropods behaved normally. Without putting them in a container, it is impossible to measure their respiration. Inclusion of elements like sediment would also introduce sources of error due to sediment respiration, etc., which are obviously undesirable. The impact of the confinement is difficult to examine but we will acknowledge this limitation in the revised version.

My more specific comments are as follows: The methods appear quite straightforward, but some additional information should be provided. Specifically: 1) What was the potential for dilution of gas within each chamber with air entering through the hole in the foil and/or around the edge of the foil (was this sealed in place)?

Response: The small hole (diameter: 2mm) on the lid is designed to keep the pressure balance between the inside and outside of the containers but will not result in abrupt air exchange. The small hole used for ventilation has been demonstrated in the previous studies (e.g. Carleton et al. 2004).

2) How many animals of each taxa were used in each experiment? How many animals were in each chamber?

Response: There were 15 replicates for each taxon in experiment 1, 10 replicates for each group in experiment 2, 15 replicates for each group in experiment 3, and one animal was put in each chamber each time to measure its CO_2 efflux in experiment 4. These numbers will be included in the revised version.

3) Ln 143: Presumably the MPB fed to the fauna were obtained through sieve and spin, otherwise what was the source of MPB? If MPB were from sieve and spin, was the MPB confirmed to still be living? How was the MPB provided, given that the final step was concentration on a filter?

Response: Yes, the MPB fed to the fauna were obtained via the sieve and spin method. When we checked the MPB in the top layer of the separated solution under microscope,

we can observe the motility of the MPB. The MPB was collected on pre-combusted GF/F filters, which were put in the containers and the benthos feed on the MPB on the filters.

4) Was experiment 4 run separately to the other experiments? Were animal sizes standardised in the other experiments?

Response: Yes, experiment 4 was run separately. We standardized animal sizes (line 187-8).

5) CH4 analysis is mentioned, but no data is presented.

Response: CRDS can simultaneously measure CO2 and CH4 concentrations and isotope values but CH4 concentrations are too low to build up in the container and are therefore not used in our analysis. We shall clarify this in the revised ms.

6) How many leaf and MPB samples were analysed? What area were these collected from? Where were these collected vs where were animals collected?

Response: We collected senescent leaves in one zip-lock bag (23 $\times$15cm), and 10 bags of surface sediments to separate the MPB. The samples were collected from Ting Kok mangroves, Hong Kong. The animals were collected from Ting Kok and Mai Po mangroves (Line 110-1).

7) Crab tissues were apparently analysed after incubation, but this data is not presented. It would be interesting to see how d13C values for muscle and other tissues compared to d13C-CO2resp. Were gastropods analysed for tissue d13C?

Response: We have shown the comparison of δ13C-CO2 respiration for crabs with classic tissue isotope analysis but found no significant difference between them (Line 235-7). Gastropods were not analysed for tissue δ13C.

8) Confirm sampling times: 20 minutes after incubation began then every 10 minutes over 50 additional minutes?

Response: It is 20 minutes after incubation began then every 10 minutes over 40 additional minutes. Twenty minutes after incubation is the start point (0 minutes) and the sampling sequence is 0, 10, 20, 30, 40 minutes. We shall replace 50 minutes (Line 139) with 40 minutes in the text.

Throughout the ms, replace lower/higher with 13C-depleted/13C-enriched (or similar)

Response: In our manuscript, we compare δ13C-CO2 for animals of different taxa, feeding habits and regime. “Lower/higher” are the preferred terminology for describing δ13C values to “depleted/enriched” (see Bond and Hobson 2012). Where appropriate we shall use “13C-depleted/13C-enriched” as descriptors (but not for δ13C values).

It would make reading easier if the authors replaced “d13C of deposit feeders respired CO2”, which is grammatically awkward, with “d13C-CO2resp for deposition feeders”. This abbreviation (or similar) could be used throughout the ms to improve readability.

Response: We will make the change throughout the ms.

Ln 14-16 and elsewhere: It is not immediately clear what is meant by “feeding regime”

Response: We shall explain it in parentheses.

Ln 26: “on field collection” – it is unclear what this means without having read the remainder of the paper

Response: We shall explain it in parentheses.

Ln 39: Remove ‘past’

Response: Agreed.

Ln 47 onwards: Rework. The focus here on predators seems at odds with the focus of this manuscript on detritivores and deposit feeders.

Response: Agreed. We shall make the change to suit our focus.

Ln 48: Provide references for previous use of d13C-CO2 techniques

Response: We shall add references for the previous use of δ13C-CO2 techniques, including Engel et al. (2009) and Carleton et al. (2004).

Ln 75-76: Unclear.

Response: We mean “few studies explore whether the increase in CO2 emission rates from sediment surface of mangroves is related to the feeding regime or the feeding habit of the benthos when they are included”.

Ln 125-130: Some of this information would be better placed in the introduction

Response: Agreed. We shall include this information in the Introduction section.

Ln 126: Apparently crabs and gastropods were compared, but it seems likely there could be just as much different between the crab groups (ocypodids vs sesarmids) as between crabs and gastropods.

Response: There may be similar pattern on the differences. Ocypodids and sesarmids have been included as deposit feeders and detritivores in our analysis, respectively. We have compared the difference between crabs and gastropods (Line 221-3) as well as between deposit feeders and detritivores (Line 223-5), and thus have not directly compared the difference between ocypodids and sesarmids.

Ln 140: remove one mention of “collected”

Response: Agreed.

Ln 143: Unclear what is meant by “gas samples were collected separately”.

Response: We mean gas samples were collected each time when the crabs were fed on different foods.

Ln 153-154: Presumably the samples were homogenised. Were the crabs dissected to remove tissues first?

Response: No, these groups of crabs were dried and then their weight measured.

The other group of crabs were dissected to remove tissues (Line 155-6) for isotopic analysis. Otherwise, if the tissues were removed first, the final weight of the crabs will be underestimated.

Ln 197&Ln 200: Specify what the groups were (e.g. crabs vs gastropods, or different crab groups vs gastropods?)

Response: Agreed. We shall show the groups as the reviewer indicated.

Ln 218-219 seems repeated in ln 219-220.

Response: We shall rearrange the sentences to avoid repeating the same information.

Ln 227-230: Somewhat misleading. The pattern is consistent, but the magnitude is different.

Response: We shall supplement the sentence to show there is a difference in magnitude.

Ln 245 (and elsewhere): reduce repetition, “CO2 production significantly increased with carapace length” (remove “there was a significant relationship. . .”_

Response: Agreed. We shall revise the sentences to avoid repetition in the context.

Ln 249-250: Sentence is unclear.

Response: We mean the application of the above relationship for intraspecific comparison may depend on the size measurement used. We shall correct the typo in the sentence.

Ln 259: It is not clear what is meant by ‘categories’.

Response: We shall revise the latter part of the sentence to ‘. . .to infer different benthos foods being used’.

Section 4.1: The point of this section is unclear. It seems to mainly repeat the results, with no new inferences apparent. Can the authors specifically explain how d13C-

CO2resp can indicate dietary sources in some useful way?

Response: Agreed. We shall explain this point as shown in our response to the review's main concern 2.

Ln 294: The relevance of the similarity of d13C for fasting crabs and those fed on leaves is unclear

Response: We have shown that the similar δ13C-CO2 for fasting crabs and those fed on leaves. Our result is supported by those of another study (Passey et al. 2005) (Line 296-7). It is likely some crabs (collected from the field) fed on both leaves and MPB, which have higher δ13C (-24.8 $\pm$0.6 ‰ than leaves (-26.6 $\pm$0.3 ‰. After fasting, crabs metabolise stored C which have lower δ13C than leaves. The higher δ13C in their recent food and lower δ13C in the stored C results in the δ13C-CO2 for crabs similar to those fed on leaves.

Ln 297: Interesting that fractionation of d13C-CO2resp vs d13C-diet for crabs is similar to the range here, but gastropods seem to have far greater fractionation. Why? Where the diet of crabs was switched, is it possible that they were still using stored C (e.g. lipids), and this would have diluted the d13C value of the respired CO2 and affected results seen here?

Response: One possibility is that the different fractionation between crabs and gastropods arises from the differences in the amount of methane production by microorganisms in the digestive tract (Passey et al. 2005). Yes, the use of stored C when diet shifts would dilute the δ13C-CO2.

Ln 327: Specify that C. perspicillata is a bat, and check the spelling of the species name.

Response: Agreed. We shall specify this point.

Ln 334: Check spelling of detritivore

Response: Agreed. We shall correct the typo.

Figure 1: There is one purple point in among the blue points – is this an error?

Response: Yes, we will remove it. Below is the modified figure.

Figure 4: The order of the bars (left to right) appears non-intuitive and does not match Figure 3. Also, the colours in this and other figures is unnecessary.

Response: We shall rearrange the bars to match Figure 3 and use the same colour for all the bars. Below is the modified figure.

Table 1: Is it possible to provide an error estimate for the fractionation values? E.g. d13C of individual animals – d13C of diet?

Response: Yes, we will add the standard error to the fractionation values. Below is the modified table.

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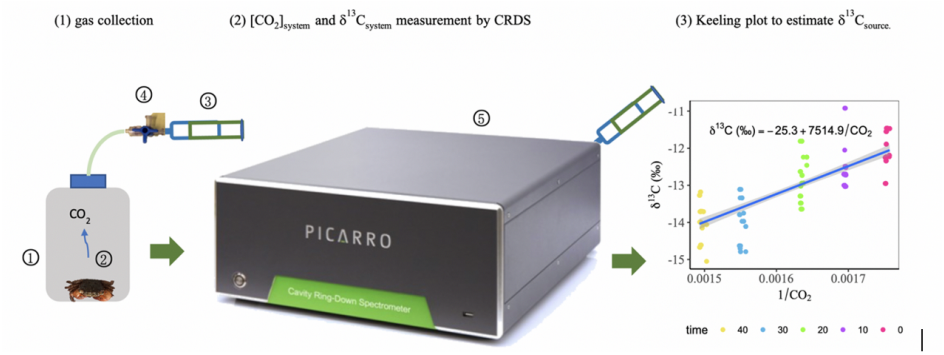

Fig. 1. Figure 1

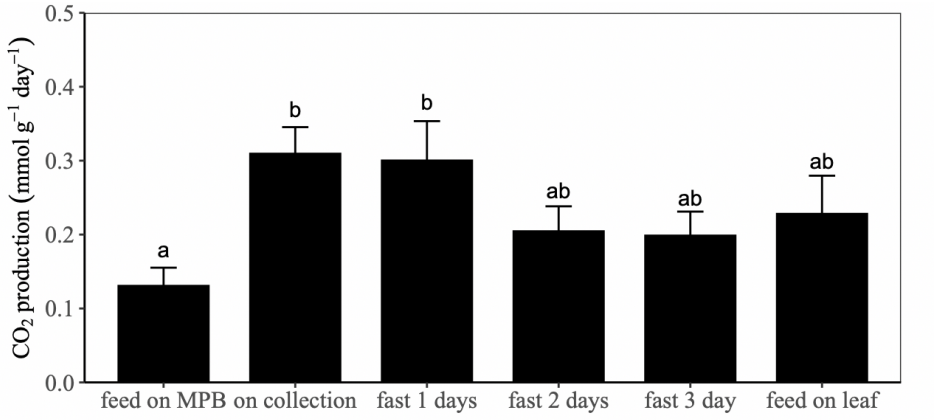

Fig. 2. Figure 4

Table 1

Benthos taxa		Diet		Isotopic fractionation	
$\delta^{13}C$-CO_2 respiration for crabs	$\delta^{13}C$-CO_2 respiration for gastropods	$\delta^{13}C$ of MPB	$\delta^{13}C$ of yellow leaves	$\Delta^{13}C_{benthos\text{-}MPB}$	$\Delta^{13}C_{benthos\text{-}leaves}$
-23.9±0.4‰	-17.5±1.3‰	-27.1±0.05‰	-27.8±0.2‰	3.2 ±0.04‰[a], 9.6 ±0.35‰[b]	3.9 ±0.06‰[a]
Feeding habit		Diet		Isotopic fractionation	
$\delta^{13}C$-CO_2 respiration deposit-feeders	$\delta^{13}C$-CO_2 respiration detritivores	$\delta^{13}C$ of MPB	$\delta^{13}C$ of yellow leaves	$\Delta^{13}C_{deposit\text{-}feeder\text{-}MPB}$	$\Delta^{13}C_{detritivore\text{-}leaves}$
-19.8±0.8‰	-24.7±0.3‰	-27.1±0.05‰	-27.8±0.2‰	7.3 ±0.14‰	3.1 ±0.07‰

[a] crab, [b] gastropod

Fig. 3. Table 1