Response to referees' comments on "Temperature-mediated changes in microbial carbon use efficiency and ^{13}C discrimination" by CA Lehmeier, F Ballantyne IV, K Min and SA Billings.

We thank the anonymous referees for their time and their efforts to improve the science and the presentation of our work. Below, we respond to each of the referees' comments (which are in italics). Please note that because of the structure of the Biogeosciences Discussions review process, we do not provide a revised manuscript at this stage but instead provide an indication of where and how we will revise the paper, if given the chance.

Anonymous referee #2:

General comments:
"Temperature-mediated changes in microbial carbon use efficiency and 13C discrimination" by Lehmeier and co-authors is a well-written manuscript that addresses questions of interest to a wide range of BG readers. The authors used stable isotope tracers and a flow-through chemostat with a single species and single carbon substrate to identify temperature controls on microbial carbon use efficiency as well as the discrimination against 13C during respiration. This study offers unique insights into the role of temperature for microbial carbon cycling and contributions to 13C-CO2 signatures.

In fact, while the authors focus on broader applications for soil and terrestrial C cycling, these results are also extremely relevant for freshwater and marine biogeochemistry and microbial ecology. Perhaps even moreso given the chemostat conditions, which may be more appropriately applied to aquatic ecosystems. The authors could reach a broader audience by acknowledging this in the language, scope, and citations of the introduction/discussion (sometimes just a matter of deleting "soil").

Response: Thank you to the reviewer for highlighting that the insights our approach reveals are simultaneously unique yet applicable to diverse systems. We agree with the reviewer that the relevance of our work for other disciplines should be acknowledged more in the manuscript. We will provide context from other disciplines in the introduction, and weave this idea into our discussion as well. We have selected multiple citations useful for these changes (e.g. Goldman & Dennet, 2000; Pomeroy & Wiebe, 2001; Chrzanowski & Grover, 2008; Hall et al., 2008).

The chemostat set-up and equilibrium assumptions are very clearly described. I do believe the authors could be more up-front about the unknowns associated with equal labeling of cellulose and glucose within the labeled cellobiose substrate, and what this might mean for the interpretation of the discrimination results (there are hints of this in EEA methods and results/discussion, but this seems to be an unknown with significant consequences for results).

Response: The $\delta^{13}C$ value of repeated measurements of the cellobiose substrate was -24.2‰ with little variation (±0.04 1SE, n=12, Fig. 4), showing that the substrate was a homogeneous mixture. (at one stage in the manuscript, we wrote -22.4‰ for cellobiose $\delta^{13}C$; this was a typo that will be changed.) Measured against VPDB standard, the $\delta^{13}C$ of -24.2‰ implies a $^{13}C/^{12}C$ ratio in the sample of ~1/91.

Considering the molecular formula of cellobiose $C_{12}H_{22}O_{11}$, this means that not more than about one out of eight cellobiose molecules in the supplied substrate had a ^{13}C atom incorporated.
We have not determined at which positions a cellobiose molecule may have had that ^{13}C atom incorporated. We have, however, confirmation from the supplier (Sigma-Aldrich) that the cellobiose we used was of biogenic origin. Evidence from work on carbon isotope distribution within carbohydrate molecules (e.g., Rossmann et al., 1991; Gleixner & Schmidt, 1997; and references therein) suggests non-random distribution of ^{13}C in biological molecules, probably mainly caused by kinetic isotope effects in enzymatic reactions (see also Tcherkez at al., 2012). Based on this phenomenon, the ^{13}C atom in a ^{13}C-containing cellobiose molecule we supplied to the microbes will not be randomly distributed within the molecule but rather consistently on the same position. In our view, this rules out the possibility that any changes in the ^{13}C distribution within the cellobiose substrate supplied to the microbes were responsible for the $\delta^{13}C$ patterns in biomass and respired CO_2 we observed (Fig. 4).

There are still unknowns in isotope effects during glycolysis and respiration (Tcherkez et al., 2012), but considering the literature that certain enzymatic reactions in metabolic pathways discriminate against ^{13}C in organic molecules in a predictable way, we believe that our discussion offers parsimonious explanations for the observed isotope patterns in biomass and respired CO_2. In a revision, we will include these considerations.

Are there recommendations for how these results can be applied to non-steady state scenarios in heterogeneous soil or biofilm matrices? This study is novel and useful, but drawing connections from steady-state chemostat measurements to the real world remains a challenge.

Response: We agree with the reviewer in that it would be very interesting to have more information about how microbial C and C isotope fluxes during the transformation of organic matter change with environmental/growth conditions.

In our experiments, maintaining microbial growth in steady-state was critical for permitting direct measurement of respiration rates and the $\delta^{13}C$ of respired CO_2, without the confounding influences that measurements in less controlled environments entail. The steady-state growth was hence a means to obtain data that would otherwise be only very difficult if not impossible to obtain, and to study the effect of temperature on microbial C and C isotope fluxes in "isolation." That is, we strived to maintain all other environmental factors constant except temperature.

Microbial experiments in controlled environments always reveal only a snapshot of specific growth and environmental conditions, and accurate generalizations therefore are difficult to make. However, we have no reason to assume that the principles and mechanisms about the temperature response of C and C isotope fluxes identified in our work wouldn't happen in the "real world" (like, a heterogeneous soil or an aquatic environment). Because our work used a simplified system to explore fundamental microbial respiration rates and respiratory C isotope discrimination during organic matter transformations with varying temperature, it serves as a starting point from which we can formulate new hypotheses, and explore, for instance, the reason for the presumably strong discrimination against ^{13}C-containing cellobiose molecules in the supplied substrate.

Specific comments:
Page 17372, line 15 - Is a 1:1 respiratory quotient appropriate for both cellulose and glucose? I would guess cellulose RQ > 1, while glucose RQ_ 1.

Response: From our data we obtained and discussed some circumstantial evidence that Cellobiose was cleaved intercellularly, and that the cleavage products, two Glucose units, then could serve as substrate for respiration. Considering that the group of carbohydrates is generally ascribed an RQ of 1, we used an RQ of 1 at that point of the manuscript to highlight that the supply rate of O_2 to the reactor should have exceeded the consumption of O_2 by the microorganisms by far, and thus allow for aerobic metabolism of the population in the reactor. If cellobiose had an RQ > 1, then the molar amount of O_2 consumed would be lower than the molar amount of CO_2 produced, which should support the point of aerobic metabolism in our chemostat reactors even more.

Figures –What is the uncertainty of the results presented in Figures 3, 4, 6?

Response:
The data presented in these figures are from seven independent chemostat runs, conducted at seven different temperatures. Therefore, we were unable to provide error bars that would indicate any variation across true experimental replicates. However, in a revision, we could provide measurement uncertainties for the concentration and $\delta^{13}C$ of respired CO_2, based on large numbers of measurements of reactor headspace CO_2 concentrations and $\delta^{13}C$ at steady-state.

(please see also below our response to the referee #3 critique to the lack of replicates.)

Figure 5 could more clearly identify the knowns/unknowns beyond boxed and unboxed. Perhaps two panels to show the difference in (a) steady-state chemostat versus (b) soil measurements and the unknowns/challenges for moving forward?

Response: In a revision, we will modify Figure 5 in response to this comment so that differences between steady-state chemostat vs. soil measurements and the unknowns/challenges for moving forward become clearer.

Anonymous referee #3:

This manuscript reports on results from a series of chemostat-based (at steady state) measurements of respiration and d13C of CO2 from cultures of P. fluorescens at various temperatures. The objective is to study the temperature dependence of microbial CUE and C isotope discrimination. It is clear that solid measurements of CUE are very difficult and thus the understanding of the factors that influence it. It is also clear that the microbial fractionation of C isotopes is a big unanswered question that keeps us

from being able to use it to help us understand the C cycle. I applaud the effort by the investigators to try to tackle these very difficult questions and think that there is promise in the approach. However, I see a fair number of aspects that add too much uncertainty to the findings and their interpretations.

The points I consider more critical are explained below and are followed by other secondary but also important issues. SGR, SRR, CUE data: CUE is defined as the fraction of SGR out of the sum of SGR and SRR. By definition, the reactor dilution rate is the SGR . (There is not a reference to support this approach).

Response: We appreciate the referee's thoughtful responses to our manuscript. We would like to highlight here that on pages 17373 and 17374 of the submitted manuscript we provide references to Bull (2010) and Ferenci (2008), in which multiple aspects of continuous culture experiments are described, including the notion that the dilution rate of the reactor equals the growth rate of the microbial population at steady-state. In crafting our manuscript, we attempted to find a balance between repeating the theory already established from these and many earlier papers and the application of the concept in our experiments to our readers; perhaps we erred in being too conservative with our explanations.
In a revision, we will add the citations of Egli (2015) and Dawson (1974) which are further sources of information to continuous culture techniques, as well as a reference to Smith & Waltman (1995) who provide a lucid description of the theory underlying chemostat dynamics. We also will rephrase our sentences describing how dilution rate of a reactor equals microbial growth rate at steady state for greater clarity.
Importantly, we had highlighted at several stages in the manuscript that our CUE calculations do not take into account any possible exudation of organic compounds (see also below), particularly via Figure 5 and in the text. We discuss this omission in the text. We also highlight that this formulation permits comparison with inferences about CUE in the soils literature that emanate from less well-controlled environmental samples and that use the same formulation.

The steady state of the culture is maintained by maintaining the SGR. Because SGR is maintained constant, CUE will always be explained by changes in the SRR (CO2 rate per unit of microbial biomass). Is it really possible to independently assess effects of temperature on specific growth rates, when they will be inevitably determined by the respiration rate and the microbial biomass? Thus is it really possible to estimate CUE?

Response: We agree with the reviewer that CUE of the continuous culture at steady-state, defined as SGR/(SGR+SRR), will always be explained by changes in SRR, when SGR is kept constant.
We would like to highlight that we intentionally took advantage of the established principles of continuous culture techniques, that it is the dilution rate of the reactor medium with fresh tank medium that determines the specific growth rate of the microbial population (see above and the cited references).
At steady-state, as documented by constant reactor headspace CO_2 concentrations, we can thus quantify temperature effects on metabolic/physiologic C partitioning i.e., the proportion of substrate respired per unit time (SRR) vs. the proportion incorporated into biomass per unit time (SGR). Measurements of SRR allowed us to compute the parameter CUE, as it is often done in the literature. Because we use specific respiration rates, temperature effects on respiration rates of the populations and on microbial biomass contents in the reactor at steady-state are both considered.

The authors state that "The 50% reduction in steady state dry microbial biomass with increasing temperature was due to 2.5 fold increase in SRR". In my view, given the nature of the thermostat system, the result is rather that SRR increased with temperature due to the decrease in microbial biomass with temperature. There's a circularity that complicates the interpretation of these variables when combined. In my view, the relationship of SRR and microbial biomass with temperature can be explored with more confidence than CUE and it is valuable that it was done at steady state.

Response: We agree with the referee that the wording "the 50% reduction in steady-state microbial biomass with increasing temperature was due to a 2.5 fold increase in SRR" may be overly constraining cause and effect. Given that logic, we would also like to avoid saying that the increase in SRR is "due to the decrease in microbial biomass with temperature". In a revision, we would reword this sentence and say that the increase in SRR with temperature integrates changes in absolute respiration rates of the population as well as changes in microbial biomass with temperature.

The extent of the impact of secretion/waste on the estimation of uptake (SGR+SRR) is difficult to constrain realistically and also its variation with temperature without any measurement of what was actually in the solution after filtration. How about the contribution of the further uptake and respiration of those substrates? (on a somewhat related note: the lack of enzymatic activity in the solution may mean that the enzymes are being quickly uptaken and thus are not detectable; the current interpretation of the lack of enzymes is very speculative). Because of these uncertainties the overall interpretations and general discussion on the effect of temperature on CUE are challenging to make.

Response: We agree with the referee in that our estimation of uptake as SGR + SRR is an approximation of total uptake, as it does not take into account any possible exudates. We had aimed at making this very clear and upfront in the manuscript (pages 17377 and 17378). Our intended revisions to Fig. 5 (see comment to referee #2, above) should help enhance the clarity of this point. Nevertheless, our presentation of CUE reveals meaningful information, as it is defined in our manuscript in the same way as in many other studies from diverse disciplines, yet it incorporates direct observations of fluxes that cannot be easily measured using environmental samples, if at all.

Even if we don't have measured rates of exudation, the isotope data and published literature provide points of orientation (pages 17378 and 17379) to suggest that exudation was not the major sink for exuded ^{13}C; for this to happen, the degree of metabolic fractionation would have to be unnaturally high (as discussed on page 17379). To fully counteract the negative correlation of temperature and CUE, the exudation flux F_{SER} would have had to increase at the same magnitude with decreasing temperature as F_{SRR} decreased with decreasing temperature. From our data, and from the viewpoint of temperature effects on metabolic rates (e.g., Gillooly et al., 2001) this seems very unlikely.

We cannot rule out that the microbes may have taken up secreted C-based metabolic compounds, although this does not appear to be an energetically favorable resource use strategy of the microbes in these continuous culture conditions. Considering the points above, if such a "recycling flux" would have

occurred, its effect on the overall CUE estimation should be little, as it would represent a continuous flow of C excreted from, and then re-consumed by, the microbes. Regardless, if the microbes exuded meaningful amounts of the exo-enzymes whose activity we sought, the steady-state nature of the system dictates that this exudation flux would be continuous, and hence detectable. However, whether or not the microbes exuded these enzymes does not affect any major conclusion of this work.

Isotopic discrimination data: in my view there is too much uncertainty in what happened with the C during the experiments and this is combined with various unexpected hard- to- explain observations. A full budget approach, accounting for all pools (both their size and isotopic composition), including inorganic C, dissolved organic (not cellobiose) could have potentially allowed explanations to allow confident interpretations. The uncertainty in the potential reasons for the very d13C values in the early incubations, and more importantly the reasons for the gradual change towards 'real' values is a problem. We don't know to what extent the processes at play during the 'climbing' phase are still at play during the plateau. The carbonate system explanation for the early stages would need measurement of the pool of inorganic C.

Response: We agree with the referee that more data would be helpful for constraining $^{13}C/^{12}C$ flows during the microbial transformation of cellobiose in this system. However, we would like to differentiate the two categories of data here – inorganic and organic C.

First, when designing the system, we recognized the importance of inorganic C fluxes as drivers of both the concentration and the isotopic signature of the CO_2 we measured in the reactor headspace. As such, we provided a detailed description of how the inorganic C system proceeds to chemical and isotopic equilibrium in the reactor in the Supplementary Material part of the current submission. These efforts in the Supplementary Material – including references to the literature and experimental data – clearly show that knowing the size and C isotopic composition of the inorganic C pools (other than CO_2 in reactor headspace) is not necessary for the accurate assessment of microbial respiration rates and the isotopic signature of respired CO_2 at steady-state. The Supplementary Material also offers an explanation for the gradual changes in concentration and $\delta^{13}C$ of the reactor headspace CO_2 during the "climbing phase", and that these changes do not pose any problem to our measurements of microbial C fluxes at steady-state, where all C pools (including the inorganic C pools in the reactor medium) are in chemical and isotopic equilibrium. In a revised manuscript version, we will add more references to this Supplementary Material to ensure that readers are aware that we address this issue in full.

Second, we agree with the referee that knowing the pool sizes and $\delta^{13}C$ of any microbial exudates would be ideal. Such data are not at all trivial to obtain. Nevertheless, we attempted to account for microbial exudation by measuring the $\delta^{13}C$ of reactor filtrate (Fig. 4), but unfortunately we were searching for a relatively small change in a large pool of unconsumed cellobiose, and we could not detect an increase in the $\delta^{13}C$ of the filtrate compared to the $\delta^{13}C$ of the cellobiose (see below). Such an increase would indicate what must be the presumed fate(s) of the ^{13}C in our system – isotopic enrichment of the unconsumed cellobiose in the reactor waste line, and/or isotopic enrichment of exudates. The discussion in the text describes how discrimination against ^{13}C during exudation is not a reasonable means of generating the isotopic patterns we observed in isolation from discrimination during uptake, but even knowledge of a very different $\delta^{13}C$ of the filtrate wouldn't have allowed us to parse these processes (exudation and uptake fractionation).

The observation that microbial biomass was depleted in 13C relative to the substrate is surprising given findings of previous much simpler studies. If they are depleted we would expect enrichment of the respiration, which what not the case. What accounts for the further depletion of the respiration is too hard to explain and it is seriously speculative. It is surprising that the d13C values of the filtrate are not presented and that an attempt to partition is not done. They suggest microbial discrimination against heavy (enriched) substrate. With a d13C value of -24, the atom percent of 13C is very low. Could the actual amount of potential 13C to discriminate against explain the actual degree of enrichment? Again, a budget approach would have helped here. The observation of strong fluctuations in the microbial and respiration with temperature is very (very) hard to explain and grasp and the current attempt is highly speculative.

Response: We agree that the fate of the "missing" ^{13}C in our system is not directly observed. We address this in our response to the "Isotopic discrimination" comment above. Briefly, we attempted to do what the referee calls for, and indeed present $\delta^{13}C$ values of the filtrate from four out of the seven chemostat runs in Fig. 4a of the manuscript. As described in the manuscript, the values do not differ significantly from the $\delta^{13}C$ values of the cellobiose. This reflects our estimates that roughly 95% of the cellobiose that is fed to the cultures is not consumed by the reactor populations at all temperatures, hence, the $\delta^{13}C$ value of the filtrate must be very close to that of the main organic component cellobiose (see lines 15 and following on page 17378). Thus, we were searching for a small change in a relatively large pool. Our data suggest that 1) the amount of exudates in the filtrate was low compared to the amount of cellobiose and 2) any ^{13}C enrichment of exudates (as one possible fate of the "missing" ^{13}C) would not have been large enough to make the $\delta^{13}C$ value of the filtrate significantly different from that of the cellobiose (see also our isotopic mass balance considerations on page 17379).

We agree that $\delta^{13}C$ data for organic compounds other than cellobiose in the filtrate would be most desirable and would allow us to come closer to the full budget approach the referee mentions. However, we emphasize that our experimental system provides data that are the closest our community has come to direct observations of these C and C isotope fluxes. Because there is not much comparable information in the literature, opportunities for discussion are minimal.

We contend that our work provides a robust example for how science progresses: it produces novel data that have not been presented in such a manner before, and it offers both novel information for researchers and hypotheses for further experimentation. Indeed, we are currently expanding on the work by performing experiments in which we explore the reason for the presumably strong discrimination against ^{13}C-containing cellobiose molecules in the substrate.

I also wonder what would have happened if the runs had been replicated and the contribution of experimental error to the ups and downs.

Response: We agree with the reviewer that performing replicate chemostat runs would be desirable. With a certain capacity for experimentation, we decided to increase the range of independent

chemostat runs at different temperatures instead of performing a higher number of experimental replicates at fewer temperatures. This trade-off is very common in chemostat-studies, suggested by the fact that a lack of true replicates can frequently be found in chemostat literature. Though perhaps not ideal, the approach has permitted chemostat enthusiasts to draw viable conclusions about microbial populations for decades.
Importantly, we performed the chemostat runs in random order of temperature (page 17372). This means that the temperature response of specific respiration rate, CUE and $\Delta^{13}C$ (Figs. 3, 4 & 6) were not due to any systematic drifts in measurements or experimentation with time.

The combined uncertainties in the isotope data and metabolism data then make the discussion on the relationship between them a bit of a stretch.
Response: We agree with the referee that our manuscript highlights several points that deserve further exploration. We never make the claim that our results are ubiquitous. However, rather than highlighting the lack of complete answers, we agree with the referee in that there is promise in our novel approach. Please see our responses to the above concerns about our statements on isotopic discrimination and CUE.

Introduction -More background on the connection between metabolism and isotopic discrimination would be nice to have. -There's not sufficient background on the factors that may drive microbial C discrimination.

Response: In a revised version, we will aim to satisfy this comment in the Introduction and provide a bit more information about C isotope discrimination and metabolism (see also the responses to referee #2, above). However, we will refrain from expanding on this point in the discussion given that we do not provide data showing metabolic fluxes.

Methods -what is the material of the filters? Could filter adsorb some molecules that the "removal" of material from the filter would not get?
Response: The material of the filters we used was polyethersulfone, and we will add this information to the methods section. For the collection of dry microbial biomass for elemental analysis, we did not aim at removing all microbial biomass from a filter, as this could have potentially contaminated the biomass with filter material and hence affected measured C contents and $\delta^{13}C$ of microbial biomass. For the determination of microbial dry weight on a filter, we subtracted the mass of the dry, clean filter from the mass of the dry filter including dried microbial biomass. Whether there was microbial biomass adsorbed by the filters or not is hence irrelevant for the estimation of microbial dry weight on a filter. In a revision, we will amend the section accordingly.

Cited literature:

Bull, A.T.: The renaissance of continuous culture in the post-genomics age, J. Ind. Microbiol. Biotechnol., 37, 993-1021, 2010.

Chzanowski, T.H., and Grover, J.P.: Element content of Pseudomonas fluorescens varies with growth rate and temperature: A replicated chemostat study addressing ecological stoichiometry, Limnol. Oceanogr., 53, 1242-1251, 2008.

Cotner, J.B., Makino, W., and Biddanda, B.A.: Temperature affects stoichiometry and biochemical composition of Escherichia coli, Microb. Ecol., 52, 26-33, 2006.

Dawson, P.S.S.: Microbial Growth, Dowden, Hutchinson & Ross, Stroudsburg, 1974.

Egli, T.: Microbial growth and physiology: a call for better craftsmanship. Front. Microbiol., 6:287, DOI: 10.3389/fmicb.2015.00287, 2015.

Ferenci, T.: Bacterial physiology, regulation and mutational adaption in a chemostat environment, Adv. Microb. Physiol., 53, 169-230, 2008.

Gillooly, J.F., Brown, J.H., West, G.B., Savage, V.M., Charnov, E.L.: Effects of size and temperature on metabolic rate, Science, 293, 2248-2251, 2001.

Gleixner, G., and Schmidt, H.-L.: Carbon isotope effects on the Fructose-1,6-bisphosphate aldolase reaction, origin for non-statistical ^{13}C distributions in carbohydrates, J. Biol. Chem., 272, 5382-5387, 1997.

Goldman, J.C., and Dennet, M.R.: Growth of marine bacteria in batch and continuous culture under carbon and nitrogen limitation, Limnol. Oceanogr., 45, 789-800, 2000.

Hall, E.K., Neuhauser, C., and Cotner, J.B.: Toward a mechanistic understanding of how natural bacterial communities respond to changes in temperature in aquatic ecosystems. ISME J., 2, 471-481, 2008.

Pomeroy, L.R., and Wiebe, W.J.: Temperature and substrates as interactive limiting factors for marine heterotrophic bacteria. Aquat. Microb. Ecol., 23, 187-204, 2001.

Rossmann, A., Butzenlechner, M., and Schmidt, H.-L.: Evidence for a nonstatistical carbon isotope distribution in natural glucose, Plant Physiol., 96, 609-614, 1991.

Smith, H., and Waltman, P.: The theory of the chemostat: dynamics of microbial competition. Cambridge Unversity Press, 1995.

Tcherkez, G., Mahé, A., and Hodges, M.: 12C/13C fractionations in plant primary metabolism. Trends Plat Sci., 16, 499-506, 2012.