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.

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Title: Temperature-mediated changes in microbial carbon use efficiency and ^{13}C discrimination

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Abstract

Understanding how carbon dioxide (CO_2) flux from ~~soils~~ ecosystems feeds back to climate warming depends in part on our ability to quantify the efficiency with which microorganisms convert ~~soil~~ organic carbon (C) into either biomass or CO_2. Quantifying ecosystem-level respiratory CO_2 losses often also requires assumptions about stable C isotope fractionations associated with the microbial transformation of ~~soil~~ organic substrates. However, the diversity of organic substrates' $\delta^{13}C$ and the challenges of measuring microbial C use efficiency (CUE) in their natural environment ~~soils~~ fundamentally limit our ability to project ~~soil, and thus~~ ecosystem~~,~~ C budgets in a warming climate. Here, we quantify the effect of temperature on C fluxes during metabolic transformations of cellobiose, a common microbial substrate, by a cosmopolitan ~~soil~~ microorganism growing at a constant rate. Specific respiration rate increased by 250% between 13 °C and 26.5 °C, decreasing CUE from 77% to 56%. Specific respiration rate was positively correlated with an increase in respiratory ^{13}C discrimination from 4.4‰ to 6.7‰ across the same temperature range. This first demonstration of a direct link between temperature, microbial CUE and associated isotope fluxes provides a critical step towards understanding $\delta^{13}C$ of respired CO_2 at multiple scales, and towards a framework for predicting future ~~soil~~ ecosystem C fluxes.

1 Introduction

Because Earth's C cycle is a key regulator of climate, a central goal of biogeochemistry is to understand ~~terrestrial~~ biosphere-atmosphere C exchange. Globally, almost all C initially

assimilated via photosynthesis is respired ~~by plants and soil microorganisms~~ back to the atmosphere as CO_2 by auto- and heterotrophic organisms (Schimel, 1995; Trumbore, 2006). Though we have a reasonably comprehensive understanding of how environmental conditions influence ~~photosynthetic~~ CO_2 uptake by ~~plants~~photosynthetic organisms, our understanding of how respiratory CO_2 fluxes respond to environmental conditions significantly lags behind. This is especially true for respiratory CO_2 derived from heterotroph~~ic soil microorganisms s in aerobic conditions~~, which may account for ~~~65%~~more than half of ~~the~~ respiratory C~~O~~$_2$ losses from soils and aquatic systems (Kucera and Kirkham, 1971; Hanson et al., 2000; Cotner and Biddanda, 2002; Subke et al., 2006). Metabolic rates of heterotrophs ~~soil microbes~~ are expected to increase with rising temperatures (Gillooly et al., 2001; Pomeroy and Wiebe, 2001; Hall et al., 2008), which is of great concern given ~~the~~ Earth's large reservoir of ~~C substrates in soil~~ reduced organic matter (~~S~~OM) that may be mineralized to CO_2 via metabolism (Hedges et al., 2000; Kirschbaum, 2006). The influence of temperature on the physiology of ~~soil~~ heterotrophic microbes must therefore be well understood to project shifts in the ~~terrestrial~~ global C balance in a warmer climate.

Existing knowledge of Earth's terrestrial C balance has been bolstered by the use of stable isotopes. A milestone for progress was when photosynthetic responses to environmental conditions were linked to differences between the stable C isotopic composition ($\delta^{13}C$) of atmospheric CO_2 and that of plant products (Farquhar et al., 1982). These differences, caused by C isotope fractionation during CO_2 diffusion into leaves and subsequent carboxylation (Park and Epstein, 1961; O'Leary, 1981), impart an isotopic fingerprint on ecosystem C pools and permit inference about C fluxes from $\delta^{13}C$ of ecosystem C pools at multiple spatio-temporal scales (Farquhar and Richards, 1984; Pataki et al., 2003; Dijkstra et al., 2004; Barbosa et al., 2010).

Recent studies remind us that respiratory C losses also leave an isotopic fingerprint on $\delta^{13}C$ values of plant tissues via respiration of substrates with distinct $\delta^{13}C$ (Bathellier et al., 2009; Brüggemann et al. 2011; Ghashghaie and Badeck, 2014), and via C isotope fractionation during decarboxylation in respiratory pathways (Werner and Gessler, 2011; Werner et al., 2011; Tcherkez et al., 2012). Though not all C isotope fractionations during metabolism are well-characterized, $\delta^{13}C$ of metabolic reaction substrates and products can vary predictably, caused by kinetic or thermodynamic isotope effects (Rossmann et al., 1991; Gleixner and Schmidt, 1997; Cleland, 2005; Tcherkez et al., 2012). Accounting for isotope effects in plant respiratory C losses improves our ability to quantify the contributions of different pools to CO_2 fluxes and thus our predictions of terrestrial ecosystem C budgets under changing environmental conditions. Using $\delta^{13}C$ of heterotrophically respired CO_2 holds similar promise, but if and how changing environmental conditions influence any fractionation factors for the fluxes associated with the liberation of C from ~~S~~OM is unknown.

Significant uncertainty about the direction and magnitude of C isotope fractionation during microbial C transformations (Bowling et al., 2008; Werth and Kuzyakov, 2010) renders quantifying microbial CO_2 fluxes in ~~terrestrial~~ ecosystems difficult. Difficulties arise because ~~soil~~ microbes in natural systems can access a diverse array of ~~SOM~~ organic substrates with distinct $\delta^{13}C$ signatures (Park and Epstein, 1961; Billings, 2006), the respiration of which influences $\delta^{13}C$ of respired CO_2. Though we know the growth rate of microbial populations influences C flux into and through biomass (Kayser et al., 2005), it is impossible to directly quantify microbial growth *in situ*. Furthermore, absence of steady state conditions over a course of ~~soil~~ CO_2 flux measurements makes the interpretation of temperature effects on the magnitude and the $\delta^{13}C$ of ~~soil~~ ecosystem respiration an even greater challenge (Gamnitzer et al., 2011;

Nickerson et al., 2013). Thus, establishing a mechanistic understanding of the links between temperature, microbial respiration rates and C isotope fractionation during substrate transformations at a fundamental level requires that we characterize these processes as temperature changes in isolation from other factors that influence microbial C transformations.

To assess the influence of temperature on microbial growth and respiration rates, we grew a widely distributed Gram-negative, heterotrophic ~~soil~~ bacterium (*Pseudomonas fluorescens*) in continuous culture bioreactors (chemostats~~; Ferenci, 2008; Bull, 2010~~) at seven temperatures ranging from 13 °C to 26 °C (Fig. 1) at reactor dilution rates of approximately 0.14 h^{-1}, which is equivalent to the relative growth rates of the microbial populations (Dawson, 1974; Smith and Waltman, 1995; Goldman and Dennett, 2000; Chrzanowski and Grover, 2008; Ferenci, 2008; Bull, 2010; Egli, 2015). We measured microbial respiration rates and $\delta^{13}C$ of respired CO_2 in this open, ~~flow~~ flow-through system at steady-state (Craig and Gordon, 1965; Fry, 2006; see Supplementary Material for a detailed elaboration of this approach). We computed the temperature dependence of a widely applied metric of microbial C use efficiency (CUE), defined as SGR / (SGR+SRR), where SGR and SRR are specific growth and specific respiration rates respectively, with units of C per microbial biomass-C and time. Our simplified system eliminates factors present in ~~real soils~~natural environments that preclude accurate assessment of specific growth and respiration rates, and thus accurate estimates of CUE as defined above. Obtaining accurate estimates of microbial CUE is critical for projecting C fluxes into the future because the particular value of CUE significantly influences CO_2 loss rates from ~~soils~~ ecosystems in models of ~~S~~OM decomposition (Allison et al., 2010; Wieder et al., 2013). Finally, simultaneously quantifying differences in $\delta^{13}C$ of organic substrate, microbial biomass and

511 respired CO_2 along a temperature gradient is critical for partitioning synoptic CO_2 measurements
512 into ~~its~~ component fluxes.
513
514 **2 Materials and Methods**
515 **2.1 Pre-cultivation of microorganisms for chemostat inoculation**
516 We pre-cultivated *Pseudomonas fluorescens* (Carolina Biological Supply, USA) in nutrient
517 solution containing 10 mM NH_4Cl, 1.6 mM KNO_3, 2.6 mM K_2HPO_4, 1.0 mM KH_2PO_4, 0.8 mM
518 $MgSO_4$, 0.2 mM $CaCl_2$, 0.1 mM $CuCl_2$, 0.04 mM $FeSO_4$, 0.03 mM $MnCl_2$ and 0.02 mM $ZnSO_4$,
519 modified from Abraham et al. (1998). The sole C source in the nutrient medium was 10 mM
520 cellobiose ($C_{12}H_{22}O_{11}$; with a $\delta^{13}C$ of ~~-22.4~~ -24.2‰); cellobiose is a disaccharide consisting of
521 two glucose molecules and a basic module of cellulose. Thus, the C to N to P atomic ratio of the
522 autoclaved, sterile nutrient solution was 100 to 10 to 3.3; its pH was adjusted to 6.5. The
523 bacteria grew for a few days in batch culture in a flask fitted with a vent for air exchange covered
524 by a 0.22 µm filter (Fisher Scientific, USA) to avoid contamination. Vessel contents were stirred
525 continuously in an incubator maintained at 10 °C.
526 **2.2 The laboratory mesocosm – the chemostat**
527 The chemostat system was composed of two 1.9 L vessels, a medium reservoir tank and a
528 reactor, each maintained on separate heating/stirring plates (Fig. 1) in separate incubators. The
529 reactor volume was on average 870 mL (Supplementary Table 1). The reservoir tank was
530 connected via a flexible tube to the reactor (Tygon E-LFL pump tubing, Masterflex, USA),
531 which in turn had an outlet tube (Fig. 1; both tubes had a 1.6 mm inner diameter). When the

chemostat was operated in "continuous culture mode" a peristaltic pump transported fresh medium to the reactor and simultaneously removed medium from the reactor at the same rate. Thus, reactor volume remained constant during all chemostat runs. The 0.22 µm filter in the reservoir tank lid allowed for pressure compensation during withdrawal of nutrient solution in the continuous flow mode. Experimental temperatures were continuously measured with a thermometer (Oakton, USA) placed in the reactor medium (Fig. 1). This thermometer was routinely compared against an internal laboratory standard mercury thermometer, before and at the end of each experiment. The reactor temperatures were adjusted with heating/stirring plate and incubator settings, and kept constant during all experimental runs.

The reactor lid had two ports for gas lines. The outlet port tube was connected to a $^{13}CO_2/^{12}CO_2$ analyzer (G2101-i, Picarro, USA) containing a pump that continuously removed air from the reactor headspace at an average rate of 0.025 L min^{-1}. A water trap (magnesium perchlorate, Costech, USA) was installed between outlet port of the reactor and the gas analyzer. The CO_2 analyzer recorded the concentration and the $\delta^{13}C$ of the reactor headspace CO_2 a~~bout ot nce every two seconds~~0.5 Hz. The reactor's inlet tube was connected to a mass flow controller (MC-50SCCM, Alicat Scientific, USA), which in turn, was connected to a gas cylinder containing CO_2-free air (Fig. 1). The mass flow controller was programmed to maintain the reactor headspace at constant atmospheric pressure; thus, the 0.025 L min^{-1} headspace air removed by the $^{13}CO_2/^{12}CO_2$ analyzer pump was instantaneously replaced with CO_2-free air flowing from the gas cylinder into the reactor medium. ~~Considering~~ Assuming (1) that 1 mol of O_2 is consumed per 1 mol of CO_2 produced in aerobic respiration, (2) a typical reactor headspace CO_2 concentration of around 2000 ppm at steady state (see Fig. 2A and below), and (3) an O_2 concentration of 21% in the air supply to the reactor, the air supply permitted continuous aerobic

555 metabolism. Routine tests with CO_2-free air in sterile chemostats were performed to ensure there
556 were no leaks in the system.

557 **2.3. The chemostat run – standardized protocol and description of events**

558 We conducted seven independent chemostat runs, at temperatures of 13, 14.5, 16, 18, 21, 23.5
559 and 26.5 °C, in random temporal order. For each of the chemostat runs, we inoculated the
560 reactor with a 10 mL aliquot of the *P. fluorescens* pre-culture and activated the flow of CO_2-free
561 air through the reactor; this was considered time 0. At the initial stage of a chemostat run, the
562 bacteria grew in batch culture, that is, there was no flow of fresh nutrient medium from the
563 reservoir tank to the reactor, and no removal of medium from the reactor (Fig. 1).

564 **2.3.1 Respiration measurements at chemical and isotopic equilibrium in the continuous**
565 **flow chemostat at steady-state**

566 At the initial pH of 6.5, inorganic C in the fresh reactor medium was mainly in the form of
567 H_2CO_3 (aq) and HCO_3^- (Stumm and Morgan, 1981). By continuously bubbling CO_2-free air into
568 the reactor, we expelled these initial inorganic C pools from the reactor medium. This was
569 evident by concentrations of reactor headspace CO_2 of virtually zero in the early stages of batch
570 culture after each run's inoculation (Fig. 2A). During the phase of rising reactor headspace CO_2
571 via respiratory activity of the exponentially growing population (Fig. 2A), inorganic C in the
572 reactor medium accrued with the increasing addition of CO_2 from microbial respiration. That is,
573 at any point in time during the phase of increasing reactor headspace CO_2 concentration, the
574 nutrient medium acted as a sink for respired CO_2. (see also Supplementary Material).

Once the respiratory activity of the growing microbial population pushed the reactor headspace CO_2 concentration above 500 ppm, we transferred the chemostat into the "continuous culture, open system" mode (Figs. 1, 2; Ferenci, 2008; Bull, 2010). The peristaltic pump henceforth transported fresh nutrient medium from the reservoir tank to the reactor at a constant rate of, on average, 118 mL h^{-1} (Supplementary Table 1), and simultaneously removed medium from the reactor at the same rate so that the reactor volume remained constant. Initial chemostat experiments indicated that when headspace CO_2 concentrations reached 500 ppm, the bacterial population was sufficiently dense to maintain itself without being washed out via ~~medium flow~~dilution. Depending on the reactor temperature, the onset of the continuous culture mode occurred between 40 h (at 26.5 °C) and 72 h (at 13 °C) after inoculation.

After the switch from batch to continuous culture, the rate of increase in reactor headspace CO_2 concentration gradually slowed because cells were continuously diluted into the waste stream (Fig. 1), and approached a phase where the CO_2 concentration became stable (Fig. 2A). At this point, bacteria grown in continuous culture had reached the phase of steady-state growth and physiology (see Ferenci, 2008; Bull, 2010). A key feature of the continuous culture chemostat relevant to our study is that at this steady-state, the constant dilution rate of the reactor (the medium flow rate divided by the reactor volume) is equivalent to the specific growth rate of the microbial culture (Bull, 2010). That is, washout of cells with the nutrient medium flow is balanced by cell division so that the size of the population in the reactor can be expected to be reasonably constant in the time frames employed here (see discussion in Ferenci, 2008; Bull, 2010).

Critically, when reactor headspace CO_2 concentrations approached the steady-state, inorganic C pools came to their respective equilibria as well (Stumm and Morgan, 1981). At this point, pools of H_2CO_3 (aq) and HCO_3^- were no longer a *net* sink for respired CO_2. As reactor headspace CO_2 concentrations reached steady state, the system supported constant microbial CO_2 production reflective of steady-state growth under constant environmental conditions, and reflected chemical equilibrium (i.e., constant size) of the dissolved inorganic C pools. Thus, the rate of CO_2 addition to the reactor headspace volume at steady-state accurately represented the CO_2 released during microbial respiration (see also Supplementary Material).

We calculated the molar CO_2 production rate of the microbial population as the product of the average molar CO_2 concentration measured by the $^{13}CO_2/^{12}CO_2$ analyzer for 5 hours at steady state (Fig. 2A) multiplied by the molar air flow rate through the reactor, which was calculated as

$$\text{Air flow (mol min}^{-1}) = 0.96 \text{ atm} * 0.025 \text{ L min}^{-1} / (0.082 \text{ atm L mol}^{-1} \text{ K}^{-1} * 296 \text{ K}),$$

with 0.96 atm and 296 K being the barometric pressure and the temperature in the lab where the experiments were performed, 0.025 L min^{-1} the average volumetric headspace flow rate through the reactor and 0.082 atm L mol^{-1} K^{-1} the gas constant.

The $\delta^{13}C$ of the reactor headspace CO_2 during the ~~early~~ earliest batch culture phase was generally very negative~~due to the inability of~~ the $^{13}CO_2/^{12}CO_2$ analyzer cannot ~~to~~ accurately measure ^{13}C and ^{12}C in very low CO_2 concentrations (Fig. 2B). The $\delta^{13}C$ of reactor headspace CO_2 became less negative as the CO_2 concentration increased (Fig. 2B). During the "climbing" phase of the reactor headspace CO_2, the $\delta^{13}C$ of the CO_2 pool was influenced by isotopic fractionation among gaseous CO_2, H_2CO_3 (aq) and HCO_3^- (Vogel et al., 1970; Mook et al., 1974; Stumm and Morgan,

1981; Szaran, 1997), because the dissolved inorganic C pools functioned as a net sink for respired CO_2. At steady-state, with constant headspace CO_2 concentrations and constant size of the dissolved inorganic C pools (see above), isotopic equilibrium was achieved, evidenced by constant $\delta^{13}C$ readings of reactor headspace CO_2 (Fig. 2B). As such, in this open system at steady-state, the $\delta^{13}C$ of the CO_2 leaving the reactor (the CO_2 measured by the analyzer) is identical to the $\delta^{13}C$ of microbial respiration (Craig and Gordon, 1965; Fry, 2006). Importantly, this principle is valid irrespective of temperature, microbial growth rate or microbial biomass in the reactor. (See Supplementary Material for an elaboration of the principle of chemical and isotopic equilibrium.)

We used the average $\delta^{13}C$ measurement of reactor headspace CO_2 over the same five hours in the stable phase employed for calculations of microbial respiration rates (see above) as the isotopic signature of CO_2 respired by the microbial culture at each temperature. Any measurements of headspace CO_2 and $\delta^{13}C$ during the climbing phase before steady-state (Fig. 2) were not used in these calculations.

For the example chemostat at 23.5 °C, the half-life of the reactor ($t_{1/2}$), i.e., the time it took until 50% of the reactor medium was exchanged with fresh tank medium, was 5.2 h (with $t_{1/2}$ = ln(2) / (medium flow rate / reactor volume); Supplementary Table 1). In a homogeneous, well-mixed system such as that employed here, 95% of the pool (i.e., the reactor) is exchanged with new medium within approximately five times the half-life. Thus, during the respiration measurements between time 70 h and 74 h (in the example time course in Fig. 2), any “leftovers” from the batch culture mode were insignificant, and the microbial culture could be considered homogeneous. This principle was applicable to all chemostat runs we performed.

After the 5-hour respiration measurements were completed, we disconnected the gas lines from the reactor, connected the mass flow controller directly to the $^{13}CO_2/^{12}CO_2$ analyzer, and replaced the CO_2-free air cylinder with a reference gas cylinder containing 1015 ppm CO_2 at a $\delta^{13}C$ of -48.9‰ (Matheson, USA). This laboratory standard gas was previously calibrated against secondary CO_2 standards (Oztech, USA) and served for any necessary corrections of the $\delta^{13}C$ of the reactor headspace CO_2 measurements. Across the seven standard measurement procedures after each individual chemostat run, the $\delta^{13}C$ measured for the laboratory standard gas showed only slight variation (1 SD = 0.16‰). CO_2 concentration measurements needed no correction; measurements of lab-internal gases with previously determined CO_2 concentrations between chemostat runs showed very stable and accurate analyzer performance.

2.3.2 Measurements of extracellular enzyme activities at steady-state

Using principles detailed by Lehmeier et al. (2013) and Min et al. (2014), we tested reactor medium for activity of the extracellular enzymes *β*-glucosidase and *β*-N-acetyl glucosaminidase across all chemostat temperatures; we never detected extracellular activity of either enzyme. The lack of extracellular *β*-glucosidase activity indicates that the sole C source, ~~of~~ cellobiose, was directly taken up by microbes and cleaved intracellularly into glucose monomers for further metabolism. The lack of extracellular *β*-N-acetyl glucosaminidase activity suggests that the inorganic N provided in nutrient medium was the sole source of N taken up by *P. fluorescens*. These findings do not rule out the possibility that *P. fluorescens* may have taken up (i.e. recycled) any exuded C-based metabolic compounds, although such a scenario in continuous culture conditions may not appear to be energetically favorable. ~~These~~ Thus, ~~inferences strengthened~~

660 ~~our~~the assumption that the sole resources used by *P. fluorescens* were the cellobiose and the
661 nutrient medium appears reasonable.

662 **2.3.3 Harvest of microbial biomass at steady-state**

663 Immediately after completing the 5 hour respiration measurements, we filtered approximately
664 300 mL of reactor medium for steady-state microbial biomass using 0.2 µm filters made of
665 polyethersulfone (Pall, USA) and a vacuum pump. The filters had previously been oven-dried
666 for 48 h at 75 °C and their dry weight determined. We then oven-dried the filters post filtration
667 for 48 h at 75 °C, removed some of the dry biomass and weighed 1.2 mg of the ~~dry~~
668 ~~biomass~~material into tin cups for subsequent combustion in an elemental analyzer (1110 CHN
669 Combustion Analyzer, Carlo Erba Strumentazione, Italy) coupled to a ThermoFinnigan DeltaPlus
670 mass spectrometer (Finnigan MAT, Germany) at the Keck Paleoenvironmental and
671 Environmental Stable Isotope Laboratory (The University of Kansas, USA). We thus determined
672 biomass C (and N) elemental content, as well as the $\delta^{13}C$ of the biomass. In this analysis, the
673 samples were compared against a laboratory standard CO_2 previously calibrated against the same
674 secondary CO_2 standards as used in calibration of the CO_2 standard used for respiration
675 measurements (see above). The $\delta^{13}C$ of the substrate cellobiose was measured likewise. Dry
676 weight of the sampled reactor aliquots and the C content obtained from elemental analysis served
677 to calculate total microbial C content in the steady-state reactor and to calculate specific
678 respiration rates.

679 At all temperatures studied, biomass C and N contents were virtually the same, on average 27%
680 and 8% of microbial dry mass, respectively (Supplementary Table 1). From mass balance
681 calculations, we determined that only a small percentage of the C and N supplied via fresh

medium from the tank was taken up to fuel microbial growth (~~1.8~~ 2.8 % and 4.3 % on average for C and N, respectively). This suggests that the observed temperature effects on specific respiration rates and CUE were not confounded by any differences in C and N limitations at the different temperatures (Goldmann and Dennet, 2000; Cotner et al., 2006; Chrzanowski and Grover, 2008).

3 Results and Discussion

For *P. fluorescens* grown in continuous culture, CUE, defined as SGR / (SGR + SRR), declined with increasing temperature, from 77% at 13 °C to 56% at 26.5 °C (Fig. 3A). Because specific growth rates were similar across the experimental temperatures (137 mg g^{-1} h^{-1}, ± 8 (1 SD); or 13.7 % h^{-1} in relative terms; Fig. 3B), the decline in CUE was due to the 2.5 fold increase of SRR with temperature, which rose from 45 mg g^{-1} h^{-1} at 13 °C to 113 mg g^{-1} h^{-1} at 26.5 °C (Fig. 3B). The decline in CUE is also evident in the ~~the~~ more than 50% reduction in steady-state dry microbial biomass with increasing temperature (Fig. 3A) ~~was driven by the 2.5 fold increase in SRR, from 45 mg g^{-1} h^{-1} at 13 °C to 113 mg g^{-1} h^{-1} at 26.5 °C (Fig. 3B)~~. For example, though SGR was approximately the same and thus the fraction of biomass replaced per time similar across all experimental temperatures (0.147 and 0.141 at 13 °C and 26.5 °C, respectively; Fig. 3B) microbes at 13 °C generated 20.9 mg biomass h^{-1} while those at 26.5 °C generated less than half the amount (9.5 mg biomass h^{-1}). –

Because we did not quantify possible C losses from the population at steady-state such as secretion of organic acids or other compounds (El-Mansi and Holms, 1989; Nanchen et al.,

2006), gross rates of steady-state cellobiose C uptake may have been slightly higher than what was calculated from the sum of SGR and SRR. However, the direct observation of *P. fluorescens*' CUE is consistent with the negative effect of increasing temperature on microbial CUE widely reported in literature for soils and aquatic ecosystems (del Giorgio and Cole, 1998; Gillooly et al., 2001; Rivkin and Legendre, 2001; Apple et al., 2006; Manzoni et al., 2012; Frey et al., 2013; Tucker et al., 2013).

Across the chemostat runs, we observed strong C isotope fractionations, which created pronounced differences in $\delta^{13}C$ between microbial biomass and the sole C substrate, cellobiose, and between microbial biomass and respired CO_2 (Fig. 4). Microbial biomass exhibited 5.5 to 10.5‰ more negative $\delta^{13}C$ values than the cellobiose and respired CO_2 was even more ^{13}C depleted, at least 4.4‰ more negative than the biomass (Fig. 4A). Because each chemostat was at steady-state, isotopic mass balance dictates that ^{13}C "missing" from cellobiose had to accumulate in another pool in the reactor. The only pool that could have been enriched with the "missing" ^{13}C was reactor DOC, which we analyzed for $\delta^{13}C$ in four out of the seven chemostat runs (Fig. 4A). Reactor DOC consisted of a large pool of cellobiose (because the rate of C consumption by the chemostat cultures was, on average, only ~~1~~2.8% of the rate of C supply) and presumably a pool of additional organic compounds (e.g. ~~such as~~ acetate). Such compounds appear to be typically secreted from microbial cells at low rates in aerobic chemostats operated at dilution rates similar to those of our runs (El-Mansi and Holms, 1989; Nanchen et al., 2006), and have been shown to be enriched in ^{13}C compared to cellular biomass (Blair et al., 1985). However, because such a small fraction of the available cellobiose was taken up by *P. fluorescens*, the fraction of total DOC comprised of secreted organic compounds was small. As a

result, ^{13}C enrichment of any microbial exudates was insufficient to enrich bulk DOC to an extent detectable by the isotope-ratio mass spectrometer (Fig. 4A).

The majority of the fractionation between *P. fluorescens* biomass and the substrate was most likely due to discrimination against ^{13}C during cellobiose uptake. If we assume that *P. fluorescens* secreted organic compounds at a rate of 10% of the sum of SGR and SRR (El-Mansi and Holms, 1989) and that the bacteria did not discriminate against ^{13}C-containing cellobiose during uptake (and thus assimilated cellobiose possessed a $\delta^{13}C$ of -24.2‰), isotopic mass balance dictates that the $\delta^{13}C$ of the C secretion flux (Fig. 5) would have to be +70‰, at minimum, across all temperatures. To our knowledge, such high metabolic discrimination against ^{13}C would be very unusual for biological systems (O'Leary, 1981). An alternative and more likely scenario is therefore that *P. fluorescens* took up less ^{13}C-containing cellobiose than was supplied as substrate, and that discrimination during uptake contributed substantially to *P. fluorescens* biomass and respired CO_2 being more ^{13}C depleted than the cellobiose supplied. This conclusion holds for all temperatures studied. If we assume for the example 23.5 °C chemostat run at steady-state (Figs. 2, 5) that *P. fluorescens* secreted organic compounds at a rate of 10% of the sum of SGR and SRR, and we further assume that the $\delta^{13}C$ of secreted compounds was 11.7‰ less negative than that of the biomass (Blair et al., 1985), that is -18.1‰, then ~~did not discriminate against ^{13}C during secretion but only during uptake,~~ the $\delta^{13}C$ of the cellobiose taken up would have been~~, on average, -34.2~~ -31.1‰, which is only a 10‰ difference from the cellobiose provided, and therefore probably a more likely scenario (Fig. 5).

Substantial ^{13}C depletion of respired CO_2 relative to microbial biomass has not, to our knowledge, been reported in other studies. With the microbial C consumption rate amounting to

only ~~1.8~~2.8% of the rate of C supply, C availability was high compared to what ~~soil~~ microbes in their natural environments typically experience (Tempest and Neijssel, 1978; Cole et al., 1988; Hobbie and Hobbie, 2013), potentially promoting enzymatic discrimination. The cellobiose $\delta^{13}C$ of -24.2 ‰ implies a $^{13}C/^{12}C$ ratio 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 included a ^{13}C atom. Faster diffusion of the isotopically lighter cellobiose molecules may have contributed to a lower probability of ^{13}C-containing cellobiose approaching bacterial membrane uptake sites, and hence, to the differences between $\delta^{13}C$ of substrate and biomass (Fig. 4A). However, isotope fractionation during diffusion – a physical process dependent on compound mass – would likely exhibit a continuous temperature response. Thus, it seems unlikely that fractionation during diffusion was the primary driver of the pronounced, discontinuous changes in the difference between substrate and biomass $\delta^{13}C$, which ranged from 5.5 to 10.5‰ (Fig. 4A). Rather, this variation, with one apparently linear part between 13 °C and 16 °C and another between 18 °C and 23.5 °C, may be explained parsimoniously by a significant, discontinuous reorganization of enzyme-mediated C fluxes into and out of bacterial cells (see Nanchen et al., 2006), induced by differences in temperature at which *P. fluorescens* was growing and the related differences in substrate uptake rates.

Evidence from work on C isotope distribution within carbohydrate molecules (e.g., Rossmann et al., 1991; Gleixner and Schmidt, 1997) suggests non-random distribution of ^{13}C in biological molecules (such as cellobiose). Based on such phenomena, it is probable that the ^{13}C atom in ^{13}C-containing cellobiose was consistently at the same position within the molecule, which rules out the possibility that any changes in the intramolecular ^{13}C distribution of cellobiose were responsible for the observed $\delta^{13}C$ patterns in biomass and respired CO_2 (Fig. 4). Hence, ~~T~~the

discontinuous pattern of $\delta^{13}C$ of respired CO_2 with temperature, similar to the pattern for $\delta^{13}C$ of the biomass (Fig. 4A), presumably reflects the downstream consequence of an upstream change in $\delta^{13}C$ of the metabolic substrate taken up and ultimately respired. However, the more negative $\delta^{13}C$ of respired CO_2 compared to that of biomass is, to our knowledge, the most direct evidence to date for ^{13}C discrimination during respiration of a heterotrophic ~~soil~~ microbe. The observation of a substantial respiratory ^{13}C discrimination corroborates inferences drawn in earlier studies (Ŝantrůčková et al., 2000; Fernandez and Cadisch, 2003) and is also consistent with plant studies reporting C isotope discrimination during dark respiration in roots (Klumpp et al., 2005; Bathellier et al., 2009; Ghashghaie and Badeck, 2014). Our observations of respiratory discrimination against ^{13}C highlight the similarity of heterotrophic, aerobic respiratory pathways, and isotope effects within them, across life's domains.

In contrast to the discontinuous relationship between biomass $\delta^{13}C$ and temperature, we observed a comparably continuous and linear increase in respiratory discrimination against ^{13}C with temperature (Fig. 4B). This increase generated a marginally positive significant (P=0.08) correlation with SRR (Fig. 6), and hence a marginally significant (P=0.07) correlation with CUE. A physiological interpretation of this finding is not straightforward, as multiple, possibly simultaneous enzymatic fractionations may have contributed to the observed $\delta^{13}C$ of respired CO_2 (Dijkstra et al., 2011; Tcherkez et al., 2012). It could simply result from a proportionally increasing flux through respiratory pathways, with associated stronger expression of ^{13}C discrimination by the enzymes involved (Tcherkez et al., 2012), or could result from increasing temperatures altering the relative fluxes through respiratory pathways (Chung et al., 1976; Wittmann et al., 2007; Dijkstra et al., 2011) such that the overall observed respiratory ^{13}C discrimination increased with temperature. This may be possible given that respiratory pathways

793 can exhibit distinct fractionation factors (Bathellier et al., 2009 and references therein) and
794 prompt different, specific C atoms to undergo decarboxylation from the two glucose units of the
795 substrate cellobiose, which contain non-randomly distributed ^{13}C atoms (Rossmann et al., 1991;
796 Gleixner and Schmidt, 1997). If relative fluxes through different respiratory pathways changed
797 with temperature, the continuous nature of the relationship between temperature and respiratory
798 ^{13}C discrimination suggests a smooth transition compared to the abrupt and discontinuous shifts
799 in apparent uptake and/or secretion discrimination described above. Future metabolic flux
800 analyses linked to isotopic approaches sensitive enough to quantify C isotopes in microbial
801 exudation will be well-suited to explore how C allocation to distinct, aerobic respiratory
802 pathways may vary with temperature and result in varying $\delta^{13}C$ of respired CO_2.

803

804 **4. Conclusions**

805 Our observations clearly show a decline in microbial CUE with increasing temperature when C
806 substrate is plentiful and demonstrate the mechanism driving it – an increase in SRR. The
807 relationship between CUE and temperature underscores the importance of incorporating variable,
808 temperature dependent SRR, which influences CUE and growth efficiency, in ecosystem process
809 models. The temperature-driven changes in SRR and respiratory discrimination against ^{13}C were
810 not independent of each other, suggesting that increasing SRR, to some degree, drives enhanced
811 C isotopic discrimination. We demonstrate that C isotope discrimination associated with
812 microbial decomposition of ~~S~~OM can impart large and variable isotopic signatures on C pools
813 typically characterized and interpreted in biogeochemical studies at any scale. To date, efforts to
814 partition flux components of net ecosystem exchange have assumed little to no fractionation

between respired substrates and the resultant CO_2. Our results suggest that this assumption must be reevaluated, and represent a first step towards an isotopically explicit, mechanistic framework for microbial C isotope fluxes in Earth system models.

Author contribution:

C.A.L. and K.M. performed the experiments; all authors contributed to all other parts and stages of the manuscript.

Data availability: The data presented in this study are available for collaborative use by anyone interested; contact the corresponding author for access to the data.

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Figure legends:

Fig. 1. Chemostat system comprised of *P. fluorescens* growing on cellobiose. Seven independent experiments were conducted, with reactor temperatures of 13, 14.5, 16, 18, 21, 23.5 and 26.5 °C; all other conditions were identical. During continuous flow, dilution rate of the reactor (mean=0.137±0.01 h^{-1} across all experiments) equals microbial growth rate. A peristaltic pump supplied fresh nutrient medium from a reservoir tank to the reactor and removed reactor medium (including biomass) at a constant rate. Headspace volume was flushed with CO_2-free air, bubbling through reactor medium and supplying microorganisms with O_2. A $^{13}CO_2/^{12}CO_2$ analyzer continuously sampled reactor headspace and measured the concentration and $\delta^{13}C$ of respired CO_2.

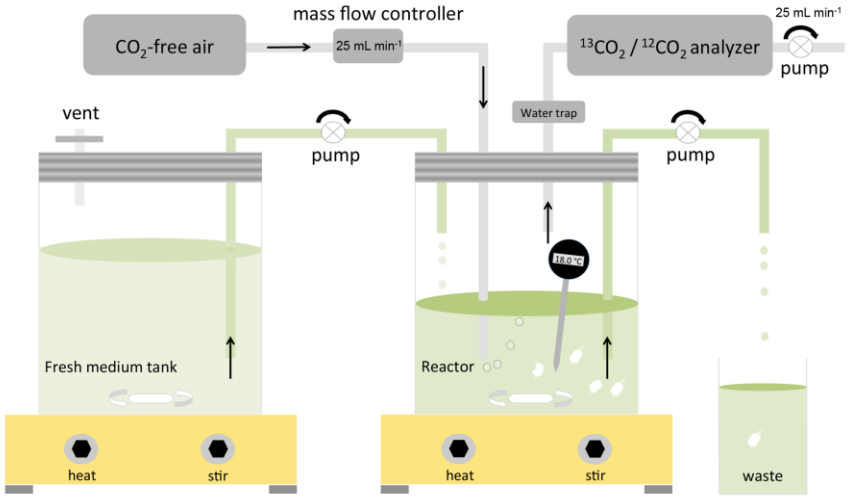

Fig. 2. Example time course of the evolution of reactor headspace CO_2 concentration (A) and $\delta^{13}C$ of the CO_2 (B) of the chemostat run at 23.5 °C in hours since inoculation of the reactor with pre-cultured *P. fluorescens*. Data points are hourly means. Error bars (where visible) denote ± 1 SD. The reactor was shifted from batch to continuous culture mode 45 h after inoculation. Microbial respiration rate and the $\delta^{13}C$ of respired CO_2 were measured between 70 and 74 h after inoculation when the culture reached steady-state.

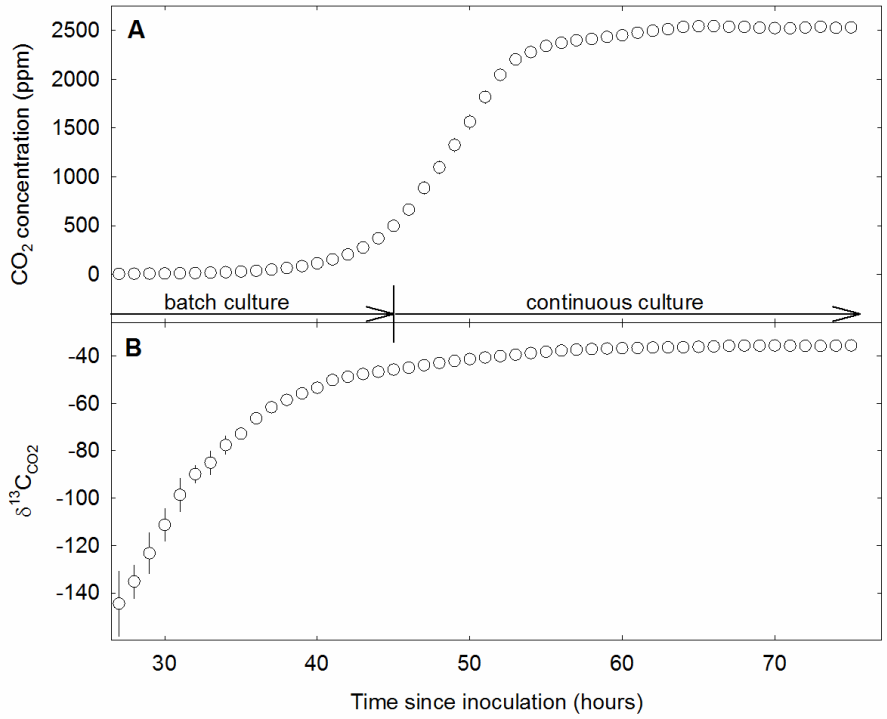

Fig. 3. Steady-state process variables of *P. fluorescens* growing in chemostats at specified temperatures. Microbial carbon use efficiency (○; A), dry microbial biomass (●; A), specific C growth rate (■; B), and specific C respiration rate (□; B), expressed per unit of microbial biomass-C**.** The dashed line denotes the average of the seven specific growth rates (137 mg g^{-1} h^{-1}).

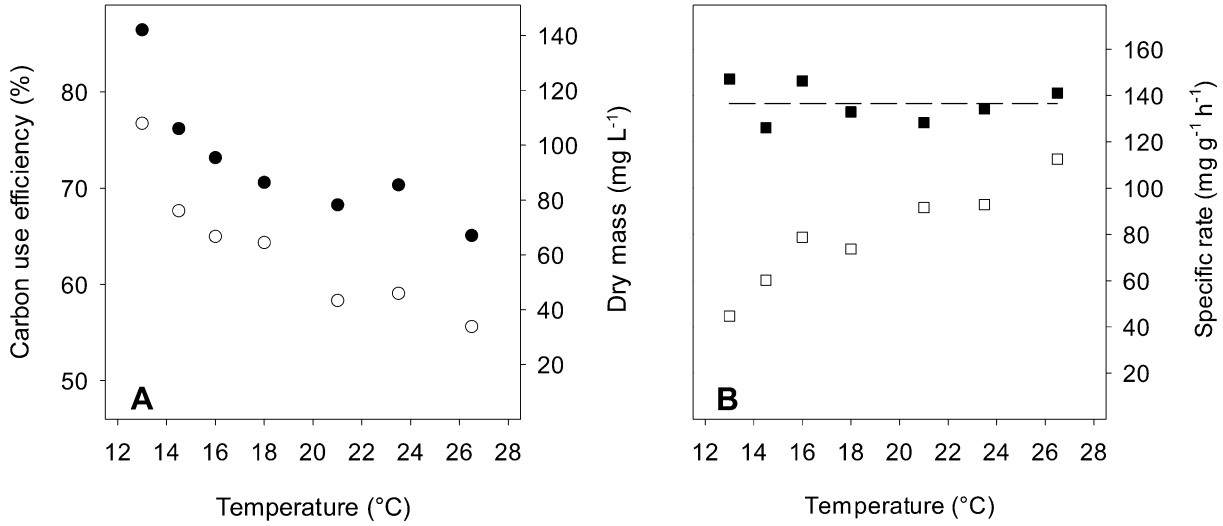

Fig. 4. Steady-state $\delta^{13}C$ of microbial biomass (●; A) and of respired CO_2 (○; A), and C isotope discrimination during respiration ($\Delta^{13}C$; B) of *P. fluorescens* growing in chemostats at specified temperatures. In panel A, the dashed line denotes the $\delta^{13}C$ of the substrate cellobiose (-24.2‰), and $\delta^{13}C$ of reactor filtrate is shown as open squares; standard errors, derived from multiple measurements across time during steady-state, are smaller than the size of the symbols. $\Delta^{13}C$ is calculated as $\Delta^{13}C = (\delta^{13}C_{biomass} - \delta^{13}C_{respired\ CO2}) / (1 + (\delta^{13}C_{respired\ CO2} / 1000))$. The solid line denotes linear regression of $\Delta^{13}C$ vs. temperature ($y = 0.14x + 3.1$; $R^2 = 0.61$; P=0.04).

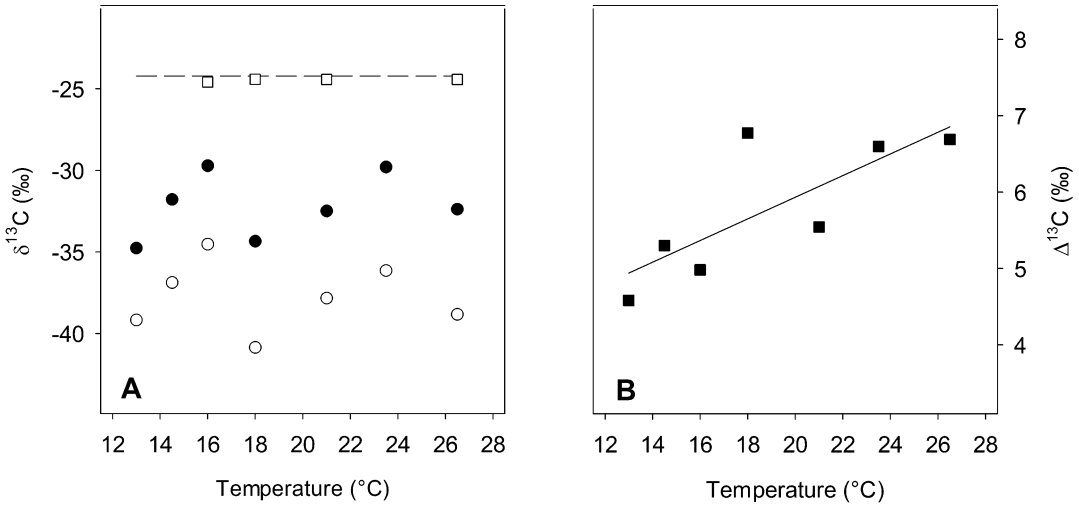

Fig. 5. Schematic of an individual *P. fluorescens* cell, representing ~~a~~ the sample population growing at 23.5 °C with one available substrate at a constant relative growth rate of 0.13 h^{-1} at steady-state (Fig. 2), with measured (solid boxed) and ~~unknown~~ estimated (dashed boxed) magnitudes of C and ^{13}C fluxes into and out of the population. Designated fluxes include specific uptake rate of cellobiose (F_{SUR}), specific growth rate (F_{SGR}), specific respiration rate (F_{SRR}) and specific excretion rate (F_{SER}), in relation to steady-state biomass-C in the chemostat, where $F_{SUR} = F_{SGR} + F_{SRR} + F_{SER}$. The estimate of $\delta^{13}C_{uptake}$ is based on the assumption that $\delta^{13}C_{exuded\ C}$ is 11.7‰ less negative than $\delta^{13}C_{biomass}$ (Blair et al., 1985) and that F_{SER} is 10% of the sum of F_{SGR} and F_{SRR} (El-Mansi and Holms, 1989). In contrast to this experimental system, in ~~soils~~natural environments~~,~~ measurements of boxed pools and fluxes ~~are~~ can be confounded by the presence of dormant microorganisms, unknown microbial growth rates, diverse available substrates, and a lack of steady-state CO_2 fluxes.

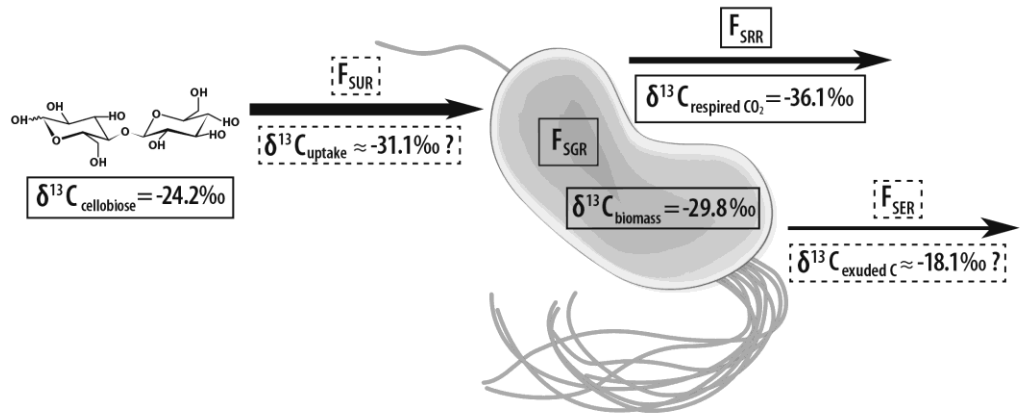

Fig. 6. Correlation between the specific respiration rate of *P. fluorescens* growing in continuous chemostat culture at temperatures ranging from 13 °C to 26.5 °C and the carbon isotope discrimination during respiration. The dashed line denotes a linear regression of the form y = 0.03x + 3.6; R^2 = 0.48; *P*=0.08.