1 Response to referees' comments on "Temperature-mediated changes in microbial carbon use

- 2 efficiency and ¹³C discrimination" by CA Lehmeier, F Ballantyne IV, K Min and SA Billings.
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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.

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10 Anonymous referee #2:

- 11 *General comments:*
- 12 *"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
- 14 readers. The authors used stable isotope tracers and a flow-through chemostat with a single species and
- 15 single carbon substrate to identify temperature controls on microbial carbon use efficiency as well as the
- 16 discrimination against 13C during respiration. This study offers unique insights into the role of
- 17 temperature for microbial carbon cycling and contributions to 13C-CO2 signatures.
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19 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
- 21 moreso given the chemostat conditions, which may be more appropriately applied to aquatic
- 22 ecosystems. The authors could reach a broader audience by acknowledging this in the language, scope,
- 23 and citations of the introduction/discussion (sometimes just a matter of deleting "soil").
- 24
- 25 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
- 27 of our work for other disciplines should be acknowledged more in the manuscript. We will provide
- 28 context from other disciplines in the introduction, and weave this idea into our discussion as well. We
- 29 have selected multiple citations useful for these changes (e.g. Goldman & Dennet, 2000; Pomeroy &
- 30 Wiebe, 2001; Chrzanowski & Grover, 2008; Hall et al., 2008).
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- 34 The chemostat set-up and equilibrium assumptions are very clearly described. I do believe the authors
- 35 could be more up-front about the unknowns associated with equal labeling of cellulose and glucose
- 36 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).
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- 40 Response: The δ^{13} C value of repeated measurements of the cellobiose substrate was -24.2‰ with little
- 41 variation (±0.04 1SE, n=12, Fig. 4), showing that the substrate was a homogeneous mixture. (at one
- 42 stage in the manuscript, we wrote -22.4‰ for cellobiose δ^{13} C; this was a typo that will be changed.)
- 43 Measured against VPDB standard, the δ^{13} C of -24.2‰ implies a 13 C/ 12 C ratio in the sample of ~1/91.

44 45 46 47 48 49 50 51 52 53 54 55 55	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 nonrandom 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 δ^{13} C patterns in biomass and respired CO ₂ we observed (Fig. 4).
57 58 59 60 61 62 63	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.
64 65 66 67 68	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.
69 70 71 72	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.
73 74 75 76 77 78 79	In our experiments, maintaining microbial growth in steady-state was critical for permitting direct measurement of respiration rates and the δ^{13} C of respired CO ₂ , without the confounding influences that measurements in less controlled environments entail. The steady-state growth was hence a <u>means</u> 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.
80 81 82 83 84 85 86 87 88 89 90	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.

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92	Specific comments:
93	Page 17372, line 15 - Is a 1:1 respiratory quotient appropriate for both cellulose and glucose? I would
94	guess cellulose RQ > 1, while glucose RQ_ 1.
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96	Response: From our data we obtained and discussed some circumstantial evidence that Cellobiose was
97	cleaved intercellularly, and that the cleavage products, two Glucose units, then could serve as substrate
98	for respiration. Considering that the group of carbohydrates is generally ascribed an RQ of 1, we used
99	an RQ of 1 at that point of the manuscript to highlight that the supply rate of O ₂ to the reactor should
100	have exceeded the consumption of O_2 by the microorganisms by far, and thus allow for aerobic
101	metabolism of the population in the reactor. If cellobiose had an $RQ > 1$, then the molar amount of O_2
102	consumed would be lower than the molar amount of CO ₂ produced, which should support the point of
103	aerobic metabolism in our chemostat reactors even more.
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107	Figures –What is the uncertainty of the results presented in Figures 3, 4, 6?
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109	Response:
110	The data presented in these figures are from seven independent chemostat runs, conducted at seven
111	different temperatures. Therefore, we were unable to provide error bars that would indicate any
112	variation across true experimental replicates. However, in a revision, we could provide measurement
113	uncertainties for the concentration and δ^{13} C of respired CO ₂ , based on large numbers of measurements
114	of reactor headspace CO ₂ concentrations and δ^{13} C at steady-state.
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116	(please see also below our response to the referee #3 critique to the lack of replicates.)
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120	Figure 5 could more clearly identify the knowns/unknowns beyond boxed and unboxed. Perhaps two
121	panels to show the difference in (a) steady-state chemostat versus (b) soil measurements and the
122	unknowns/challenges for moving forward?
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124	Response: In a revision, we will modify Figure 5 in response to this comment so that differences
125	between steady-state chemostat vs. soil measurements and the unknowns/challenges for moving
126	forward become clearer.
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130	Anonymous referee #3:
101	This manuscript reports on results from a series of observatet based (at standy state) as a series of
131	respiration and d13C of CO2 from cultures of P. fluorescens at various temperatures. The objective is to

133 study the temperature dependence of microbial CUE and C isotope discrimination. It is clear that solid

- 134 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
- 136 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
- 138 fair number of aspects that add too much uncertainty to the findings and their interpretations.
- 139
- 140 The points I consider more critical are explained below and are followed by other secondary but also
- 141 important issues. SGR, SRR, CUE data: CUE is defined as the fraction of SGR out of the sum of SGR and
- 142 SRR. By definition, the reactor dilution rate is the SGR . (There is not a reference to support this
- 143 approach).
- 144
- 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
- 152 conservative with our explanations.
- 153 In a revision, we will add the citations of Egli (2015) and Dawson (1974) which are further sources of
- 154 information to continuous culture techniques, as well as a reference to Smith & Waltman (1995) who
- 155 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 forgreater clarity.
- 158 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
- 160 5 and in the text. We discuss this omission in the text. We also highlight that this formulation permits
- 161 comparison with inferences about CUE in the soils literature that emanate from less well-controlled
- 162 environmental samples and that use the same formulation.
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166 The steady state of the culture is maintained by maintaining the SGR. Because SGR is maintained

- 167 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,
- 169 when they will be inevitably determined by the respiration rate and the microbial biomass? Thus is it
- 170 really possible to estimate CUE?
- 171
- 172 **Response:** We agree with the reviewer that CUE of the continuous culture at steady-state, defined as
- 173 SGR/(SGR+SRR), will always be explained by changes in SRR, when SGR is kept constant.
- 174 We would like to highlight that we intentionally took advantage of the established principles of
- 175 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 citedreferences).
- 178 At steady-state, as documented by constant reactor headspace CO₂ concentrations, we can thus
- 179 quantify temperature effects on metabolic/physiologic C partitioning i.e., the proportion of substrate
- 180 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.

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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

192 confidence than CUE and it is valuable that it was done at steady state.

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194 Response: We agree with the referee that the wording "the 50% reduction in steady-state microbial 195 biomass with increasing temperature was due to a 2.5 fold increase in SRR" may be overly constraining 196 cause and effect. Given that logic, we would also like to avoid saying that the increase in SRR is "due to 197 the decrease in microbial biomass with temperature". In a revision, we would reword this sentence and 198 say that the increase in SRR with temperature integrates changes in absolute respiration rates of the 199 population as well as changes in microbial biomass with temperature.

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203 The extent of the impact of secretion/waste on the estimation of uptake (SGR+SRR) is difficult to

204 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

206 of those substrates? (on a somewhat related note: the lack of enzymatic activity in the solution may

207 mean that the enzymes are being quickly uptaken and thus are not detectable; the current interpretation

208 of the lack of enzymes is very speculative). Because of these uncertainties the overall interpretations and

209 general discussion on the effect of temperature on CUE are challenging to make.

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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 ¹³C; 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.

225 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

228 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.
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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

238 full budget approach, accounting for all pools (both their size and isotopic composition), including

239 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.

242 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

- 244 of inorganic C.
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Response: We agree with the referee that more data would be helpful for constraining ¹³C/¹²C 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.

249 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

253 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 CO₂ 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 δ^{13} C of the reactor headspace CO₂ during the

258 "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

260 chemical and isotopic equilibrium. In a revised manuscript version, we will add more references to this

261 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 δ^{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 δ^{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 δ^{13} C of the filtrate compared to the δ^{13} C of the cellobiose (see below). Such an increase would indicate what must be the presumed fate(s) of the ¹³C in our system – isotopic enrichment of the

268 unconsumed cellobiose in the reactor waste line, and/or isotopic enrichment of exudates. The

- 269 discussion in the text describes how discrimination against ¹³C during exudation is not a reasonable
- 270 means of generating the isotopic patterns we observed in isolation from discrimination during uptake,
- but even knowledge of a very different δ^{13} C of the filtrate wouldn't have allowed us to parse these
- 272 processes (exudation and uptake fractionation).
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275 The observation that microbial biomass was depleted in 13C relative to the substrate is surprising given 276 findings of previous much simpler studies. If they are depleted we would expect enrichment of the 277 respiration, which what not the case. What accounts for the further depletion of the respiration is too 278 hard to explain and it is seriously speculative. It is surprising that the d13C values of the filtrate are not 279 presented and that an attempt to partition is not done. They suggest microbial discrimination against 280 heavy (enriched) substrate. With a d13C value of -24, the atom percent of 13C is very low. Could the 281 actual amount of potential 13C to discriminate against explain the actual degree of enrichment? Again, a 282 budget approach would have helped here. The observation of strong fluctuations in the microbial and 283 respiration with temperature is very (very) hard to explain and grasp and the current attempt is highly

- 284 speculative.
- 285 286

Response: We agree that the fate of the "missing" ¹³C in our system is not directly observed. We address 286 this in our response to the "Isotopic discrimination" comment above. Briefly, we attempted to do what 287 the referee calls for, and indeed present δ^{13} C values of the filtrate from four out of the seven chemostat 288 runs in Fig. 4a of the manuscript. As described in the manuscript, the values do not differ significantly 289 290 from the δ^{13} C values of the cellobiose. This reflects our estimates that roughly 95% of the cellobiose that 291 is fed to the cultures is not consumed by the reactor populations at all temperatures, hence, the δ^{13} C 292 value of the filtrate must be very close to that of the main organic component cellobiose (see lines 15 293 and following on page 17378). Thus, we were searching for a small change in a relatively large pool. Our 294 data suggest that 1) the amount of exudates in the filtrate was low compared to the amount of 295 cellobiose and 2) any ¹³C enrichment of exudates (as one possible fate of the "missing" ¹³C) would not have been large enough to make the δ^{13} C value of the filtrate significantly different from that of the 296

- cellobiose (see also our isotopic mass balance considerations on page 17379).
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We agree that δ¹³C 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.

- 301 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
- 303 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 ¹³C-containing cellobiose molecules in the substrate.
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- 311 I also wonder what would have happened if the runs had been replicated and the contribution of
- 312 *experimental error to the ups and downs.*
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- Response: We agree with the reviewer that performing replicate chemostat runs would be desirable. 314 315 With a certain capacity for experimentation, we decided to increase the range of independent 316 chemostat runs at different temperatures instead of performing a higher number of experimental 317 replicates at fewer temperatures. This trade-off is very common in chemostat-studies, suggested by the 318 fact that a lack of true replicates can frequently be found in chemostat literature. Though perhaps not 319 ideal, the approach has permitted chemostat enthusiasts to draw viable conclusions about microbial 320 populations for decades. 321 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 Δ^{13} C (Figs. 3, 4 & 6) were not 322 323 due to any systematic drifts in measurements or experimentation with time. 324 325 326 327 The combined uncertainties in the isotope data and metabolism data then make the discussion on the 328 relationship between them a bit of a stretch. 329 Response: We agree with the referee that our manuscript highlights several points that deserve further 330 exploration. We never make the claim that our results are ubiquitous. However, rather than highlighting 331 the lack of complete answers, we agree with the referee in that there is promise in our novel approach. 332 Please see our responses to the above concerns about our statements on isotopic discrimination and 333 CUE. 334 335 336 Introduction -More background on the connection between metabolism and isotopic discrimination 337 would be nice to have. -There's not sufficient background on the factors that may drive microbial C discrimination. 338 339
- 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.
- 343 provide data showing metabolic fluxes.
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347 Methods -what is the material of the filters? Could filter adsorb some molecules that the "removal" of 348 material from the filter would not get?

- 349 **Response:** The material of the filters we used was polyethersulfone, and we will add this information to
- 350 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 have affected measured C contants and S^{13} C of microbial biomass. For the
- with filter material and hence affected measured C contents and δ^{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
- 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. Ina revision, we will amend the section accordingly.
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404 Title: Temperature-mediated changes in microbial carbon use efficiency and ¹³C discrimination
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422 Abstract

423 Understanding how carbon dioxide (CO₂) flux from soils ecosystems feeds back to climate warming depends in part on our ability to quantify the efficiency with which microorganisms 424 convert soil organic carbon (C) into either biomass or CO₂. Quantifying ecosystem-level 425 426 respiratory CO₂ losses often also requires assumptions about stable C isotope fractionations associated with the microbial transformation of soil organic substrates. However, the diversity of 427 organic substrates' δ^{13} C and the challenges of measuring microbial C use efficiency (CUE) in 428 their natural environment soils fundamentally limit our ability to project soil, and thus 429 ecosystem, C budgets in a warming climate. Here, we quantify the effect of temperature on C 430 fluxes during metabolic transformations of cellobiose, a common microbial substrate, by a 431 cosmopolitan soil microorganism growing at a constant rate. Specific respiration rate increased 432 by 250% between 13 °C and 26.5 °C, decreasing CUE from 77% to 56%. Specific respiration 433 rate was positively correlated with an increase in respiratory ¹³C discrimination from 4.4% to 434 6.7‰ across the same temperature range. This first demonstration of a direct link between 435 temperature, microbial CUE and associated isotope fluxes provides a critical step towards 436 understanding δ^{13} C of respired CO₂ at multiple scales, and towards a framework for predicting 437 future soil-ecosystem C fluxes. 438

439

440 **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

443 assimilated via photosynthesis is respired by plants and soil microorganisms back to the atmosphere as CO₂ by auto- and heterotrophic organisms (Schimel, 1995; Trumbore, 2006). 444 Though we have a reasonably comprehensive understanding of how environmental conditions 445 influence photosynthetic CO₂ uptake by plantsphotosynthetic organisms, our understanding of 446 how respiratory CO₂ fluxes respond to environmental conditions significantly lags behind. This 447 is especially true for respiratory CO₂ derived from heterotrophic soil microorganisms sin aerobic 448 conditions, which may account for -65% more than half of the respiratory CO₂ losses from soils 449 and aquatic systems (Kucera and Kirkham, 1971; Hanson et al., 2000; Cotner and Biddanda, 450 451 2002; Subke et al., 2006). Metabolic rates of heterotrophs soil microbes are expected to 452 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 453 organic matter (SOM) that may be mineralized to CO_2 via metabolism (Hedges et al., 2000; 454 Kirschbaum, 2006). The influence of temperature on the physiology of soil-heterotrophic 455 microbes must therefore be well understood to project shifts in the terrestrial-global C balance in 456 a warmer climate. 457

458 Existing knowledge of Earth's terrestrial C balance has been bolstered by the use of stable

459 isotopes. A milestone for progress was when photosynthetic responses to environmental

460 conditions were linked to differences between the stable C isotopic composition (δ^{13} C) of

461 atmospheric CO_2 and that of plant products (Farquhar et al., 1982). These differences, caused by

462 C isotope fractionation during CO₂ diffusion into leaves and subsequent carboxylation (Park and

463 Epstein, 1961; O'Leary, 1981), impart an isotopic fingerprint on ecosystem C pools and permit

464 inference about C fluxes from δ^{13} C of ecosystem C pools at multiple spatio-temporal scales

465 (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 δ^{13} C 466 values of plant tissues via respiration of substrates with distinct δ^{13} C (Bathellier et al., 2009; 467 Brüggemann et al. 2011; Ghashghaie and Badeck, 2014), and via C isotope fractionation during 468 469 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-470 characterized, δ^{13} C of metabolic reaction substrates and products can vary predictably, caused by 471 472 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 473 losses improves our ability to quantify the contributions of different pools to CO₂ fluxes and thus 474 our predictions of terrestrial ecosystem C budgets under changing environmental conditions. 475 Using δ^{13} C of heterotrophically respired CO₂ holds similar promise, but if and how changing 476 environmental conditions influence any fractionation factors for the fluxes associated with the 477 478 liberation of C from SOM is unknown.

Significant uncertainty about the direction and magnitude of C isotope fractionation during 479 microbial C transformations (Bowling et al., 2008; Werth and Kuzyakov, 2010) renders 480 481 quantifying microbial CO₂ fluxes in terrestrial ecosystems difficult. Difficulties arise because soil microbes in natural systems can access a diverse array of SOM organic substrates with 482 distinct δ^{13} C signatures (Park and Epstein, 1961; Billings, 2006), the respiration of which 483 influences δ^{13} C of respired CO₂. Though we know the growth rate of microbial populations 484 influences C flux into and through biomass (Kayser et al., 2005), it is impossible to directly 485 486 quantify microbial growth *in situ*. Furthermore, absence of steady state conditions over a course of soil-CO₂ flux measurements makes the interpretation of temperature effects on the magnitude 487 and the δ^{13} C of soil-ecosystem respiration an even greater challenge (Gamnitzer et al., 2011; 488

489	Nickerson et al., 2013). Thus, establishing a mechanistic understanding of the links between
490	temperature, microbial respiration rates and C isotope fractionation during substrate
491	transformations at a fundamental level requires that we characterize these processes as
492	temperature changes in isolation from other factors that influence microbial C transformations.
493	To assess the influence of temperature on microbial growth and respiration rates, we grew a
494	widely distributed Gram-negative, heterotrophic soil-bacterium (Pseudomonas fluorescens) in
495	continuous culture bioreactors (chemostats; Ferenci, 2008; Bull, 2010) at seven temperatures
496	ranging from 13 °C to 26 °C (Fig. 1) at reactor dilution rates of approximately 0.14 h ⁻¹ , which is
497	equivalent to the relative growth rates of the microbial populations (Dawson, 1974; Smith and
498	Waltman, 1995; Goldman and Dennett, 2000; Chrzanowski and Grover, 2008; Ferenci, 2008;
499	Bull, 2010; Egli, 2015). We measured microbial respiration rates and δ^{13} C of respired CO ₂ in
500	this open, flow-flow-through system at steady-state (Craig and Gordon, 1965; Fry, 2006; see
501	Supplementary Material for a detailed elaboration of this approach). We computed the
502	temperature dependence of a widely applied metric of microbial C use efficiency (CUE), defined
503	as SGR / (SGR+SRR), where SGR and SRR are specific growth and specific respiration rates
504	respectively, with units of C per microbial biomass-C and time. Our simplified system
505	eliminates factors present in real soilsnatural environments that preclude accurate assessment of
506	specific growth and respiration rates, and thus accurate estimates of CUE as defined above.
507	Obtaining accurate estimates of microbial CUE is critical for projecting C fluxes into the future
508	because the particular value of CUE significantly influences CO ₂ loss rates from soils-
509	ecosystems in models of SOM decomposition (Allison et al., 2010; Wieder et al., 2013). Finally,
510	simultaneously quantifying differences in $\delta^{13}C$ of organic substrate, microbial biomass and

respired CO₂ along a temperature gradient is critical for partitioning synoptic CO₂ measurements
into its component fluxes.

513

514 2 Materials and Methods

515 2.1 Pre-cultivation of microorganisms for chemostat inoculation

We pre-cultivated *Pseudomonas fluorescens* (Carolina Biological Supply, USA) in nutrient 516 solution containing 10 mM NH₄Cl, 1.6 mM KNO₃, 2.6 mM K₂HPO₄, 1.0 mM KH₂PO₄, 0.8 mM 517 518 MgSO₄, 0.2 mM CaCl₂, 0.1 mM CuCl₂, 0.04 mM FeSO₄, 0.03 mM MnCl₂ and 0.02 mM ZnSO₄, modified from Abraham et al. (1998). The sole C source in the nutrient medium was 10 mM 519 cellobiose (C₁₂H₂₂O₁₁; with a δ^{13} C of -22.4 -24.2‰); cellobiose is a disaccharide consisting of 520 521 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 by a 0.22 µm filter (Fisher Scientific, USA) to avoid contamination. Vessel contents were stirred 524 525 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 532 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. 533 Thus, reactor volume remained constant during all chemostat runs. The 0.22 µm filter in the 534 reservoir tank lid allowed for pressure compensation during withdrawal of nutrient solution in 535 the continuous flow mode. Experimental temperatures were continuously measured with a 536 thermometer (Oakton, USA) placed in the reactor medium (Fig. 1). This thermometer was 537 routinely compared against an internal laboratory standard mercury thermometer, before and at 538 the end of each experiment. The reactor temperatures were adjusted with heating/stirring plate 539 540 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 ${}^{13}CO_2/{}^{12}CO_2$ 541 analyzer (G2101-i, Picarro, USA) containing a pump that continuously removed air from the 542 reactor headspace at an average rate of 0.025 L min⁻¹. A water trap (magnesium perchlorate, 543 544 Costech, USA) was installed between outlet port of the reactor and the gas analyzer. The CO₂ analyzer recorded the concentration and the δ^{13} C of the reactor headspace CO₂ about ot nce-545 every two seconds 0.5 Hz. The reactor's inlet tube was connected to a mass flow controller (MC-546 50SCCM, Alicat Scientific, USA), which in turn, was connected to a gas cylinder containing 547 CO_2 -free air (Fig. 1). The mass flow controller was programmed to maintain the reactor 548 headspace at constant atmospheric pressure; thus, the 0.025 L min⁻¹ headspace air removed by 549 the ${}^{13}CO_2/{}^{12}CO_2$ analyzer pump was instantaneously replaced with CO₂-free air flowing from the 550 gas cylinder into the reactor medium. Considering Assuming (1) that 1 mol of O_2 is consumed 551 per 1 mol of CO_2 produced in aerobic respiration, (2) a typical reactor headspace CO_2 552 concentration of around 2000 ppm at steady state (see Fig. 2A and below), and (3) an O₂ 553 concentration of 21% in the air supply to the reactor, the air supply permitted continuous aerobic 554

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

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

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

2.3.1 Respiration measurements at chemical and isotopic equilibrium in the continuous 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 the reactor, we expelled these initial inorganic C pools from the reactor medium. This was 568 569 evident by concentrations of reactor headspace CO₂ 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 via respiratory activity of the exponentially growing population (Fig. 2A), inorganic C in the 571 reactor medium accrued with the increasing addition of CO₂ from microbial respiration. That is, 572 573 at any point in time during the phase of increasing reactor headspace CO₂ concentration, the nutrient medium acted as a sink for respired CO₂- (see also Supplementary Material). 574

575 Once the respiratory activity of the growing microbial population pushed the reactor headspace CO₂ concentration above 500 ppm, we transferred the chemostat into the "continuous culture, 576 open system" mode (Figs. 1, 2; Ferenci, 2008; Bull, 2010). The peristaltic pump henceforth 577 transported fresh nutrient medium from the reservoir tank to the reactor at a constant rate of, on 578 average, 118 mL h⁻¹ (Supplementary Table 1), and simultaneously removed medium from the 579 reactor at the same rate so that the reactor volume remained constant. Initial chemostat 580 experiments indicated that when headspace CO₂ concentrations reached 500 ppm, the bacterial 581 population was sufficiently dense to maintain itself without being washed out via medium-582 583 flowdilution. 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. 584

After the switch from batch to continuous culture, the rate of increase in reactor headspace CO₂ 585 concentration gradually slowed because cells were continuously diluted into the waste stream 586 587 (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 588 physiology (see Ferenci, 2008; Bull, 2010). A key feature of the continuous culture chemostat 589 relevant to our study is that at this steady-state, the constant dilution rate of the reactor (the 590 medium flow rate divided by the reactor volume) is equivalent to the specific growth rate of the 591 microbial culture (Bull, 2010). That is, washout of cells with the nutrient medium flow is 592 balanced by cell division so that the size of the population in the reactor can be expected to be 593 reasonably constant in the time frames employed here (see discussion in Ferenci, 2008; Bull, 594 595 2010).

596 Critically, when reactor headspace CO₂ concentrations approached the steady-state, inorganic C pools came to their respective equilibria as well (Stumm and Morgan, 1981). At this point, pools 597 of H_2CO_3 (aq) and HCO_3^- were no longer a *net* sink for respired CO₂. As reactor headspace CO₂ 598 concentrations reached steady state, the system supported constant microbial CO₂ production 599 reflective of steady-state growth under constant environmental conditions, and reflected 600 chemical equilibrium (i.e., constant size) of the dissolved inorganic C pools. Thus, the rate of 601 CO_2 addition to the reactor headspace volume at steady-state accurately represented the CO_2 602 released during microbial respiration (see also Supplementary Material). 603

We calculated the molar CO_2 production rate of the microbial population as the product of the

average molar CO₂ concentration measured by the 13 CO₂/ 12 CO₂ analyzer for 5 hours at steady

state (Fig. 2A) multiplied by the molar air flow rate through the reactor, which was calculated as

607 Air flow (mol min⁻¹) = 0.96 atm * 0.025 L min⁻¹ / (0.082 atm L mol⁻¹ K⁻¹ * 296 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 Lmin^{-1} the average volumetric headspace flow rate through the reactor and 0.082 atm L mol⁻¹ K⁻¹ the gas constant.

611 The δ^{13} C of the reactor headspace CO₂ during the <u>early earliest</u> batch culture phase was generally 612 very negative; <u>due to the inability of the 13 CO₂/ 12 CO₂ analyzer <u>cannot to accurately measure</u> 13 C 613 and 12 C in very low CO₂ concentrations (Fig. 2B). The δ^{13} C of reactor headspace CO₂ became 614 less negative as the CO₂ concentration increased (Fig. 2B). During the "climbing" phase of the 615 reactor headspace CO₂, the δ^{13} C of the CO₂ pool was influenced by isotopic fractionation among 616 gaseous CO₂, H₂CO₃ (aq) and HCO₃⁻ (Vogel et al., 1970; Mook et al., 1974; Stumm and Morgan,</u> 617 1981; Szaran, 1997), because the dissolved inorganic C pools functioned as a net sink for respired CO₂. At steady-state, with constant headspace CO₂ concentrations and constant size of 618 the dissolved inorganic C pools (see above), isotopic equilibrium was achieved, evidenced by 619 constant δ^{13} C readings of reactor headspace CO₂ (Fig. 2B). As such, in this open system at 620 steady-state, the δ^{13} C of the CO₂ leaving the reactor (the CO₂ measured by the analyzer) is 621 identical to the δ^{13} C of microbial respiration (Craig and Gordon, 1965; Fry, 2006). Importantly, 622 this principle is valid irrespective of temperature, microbial growth rate or microbial biomass in 623 the reactor. (See Supplementary Material for an elaboration of the principle of chemical and 624 625 isotopic equilibrium.)

We used the average δ^{13} C measurement of reactor headspace CO₂ over the same five hours in the stable phase employed for calculations of microbial respiration rates (see above) as the isotopic signature of CO₂ respired by the microbial culture at each temperature. Any measurements of headspace CO₂ and δ^{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 631 50% of the reactor medium was exchanged with fresh tank medium, was 5.2 h (with $t_{1/2} = \ln(2)$ / 632 (medium flow rate / reactor volume); Supplementary Table 1). In a homogeneous, well-mixed 633 system such as that employed here, 95% of the pool (i.e., the reactor) is exchanged with new 634 medium within approximately five times the half-life. Thus, during the respiration 635 measurements between time 70 h and 74 h (in the example time course in Fig. 2), any "leftovers" 636 from the batch culture mode were insignificant, and the microbial culture could be considered 637 638 homogeneous. This principle was applicable to all chemostat runs we performed.

639 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 640 the CO₂-free air cylinder with a reference gas cylinder containing 1015 ppm CO₂ at a δ^{13} C of -641 642 48.9‰ (Matheson, USA). This laboratory standard gas was previously calibrated against secondary CO₂ standards (Oztech, USA) and served for any necessary corrections of the δ^{13} C of 643 the reactor headspace CO₂ measurements. Across the seven standard measurement procedures 644 after each individual chemostat run, the δ^{13} C measured for the laboratory standard gas showed 645 only slight variation (1 SD = 0.16%). CO₂ concentration measurements needed no correction; 646 measurements of lab-internal gases with previously determined CO₂ concentrations between 647 chemostat runs showed very stable and accurate analyzer performance. 648

649 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 650 medium for activity of the extracellular enzymes β -glucosidase and β -N-acetyl glucosaminidase 651 652 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 653 directly taken up by microbes and cleaved intracellularly into glucose monomers for further 654 metabolism. The lack of extracellular β -N-acetyl glucosaminidase activity suggests that the 655 inorganic N provided in nutrient medium was the sole source of N taken up by P. fluorescens. 656 These findings do not rule out the possibility that *P. fluorescens* may have taken up (i.e. recycled) 657 any exuded C-based metabolic compounds, although such a scenario in continuous culture 658 conditions may not appear to be energetically favorable. These Thus, inferences strengthened 659

660 our<u>the</u> 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 <u>completing</u> the 5 hour respiration measurements, we filtered approximately

664 300 mL of reactor medium for steady-state microbial biomass using 0.2 μm filters <u>made of</u>

665 <u>polyethersulfone</u> (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 biomassmaterial 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

biomass C (and N) elemental content, as well as the δ^{13} C of the biomass. In this analysis, the

samples were compared against a laboratory standard CO₂ previously calibrated against the same

secondary CO_2 standards as used in calibration of the CO_2 standard used for respiration

675 measurements (see above). The δ^{13} C of the substrate cellobiose was measured likewise. Dry

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.

At all temperatures studied, <u>biomass</u> C and N contents were virtually the same, on average 27%
and 8% of microbial dry mass, respectively (Supplementary Table 1). From mass balance
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 (<u>1.8-2.8</u>% 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).

687

688 **3 Results and Discussion**

- For P. fluorescens grown in continuous culture, CUE, defined as SGR / (SGR + SRR), declined 689 with increasing temperature, from 77% at 13 °C to 56% at 26.5 °C (Fig. 3A). Because specific 690 growth rates were similar across the experimental temperatures (137 mg $g^{-1} h^{-1}$, ± 8 (1 SD); or 691 13.7 % h⁻¹ in relative terms; Fig. 3B), the decline in CUE was due to the 2.5 fold increase of SRR 692 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). 693 The decline in CUE is also evident in the the more than 50% reduction in steady-state dry 694 microbial biomass with increasing temperature (Fig. 3A) was driven by the 2.5 fold increase in-695 SRR, from 45 mg g⁺ h⁺ at 13 °C to 113 mg g⁺ h⁺ at 26.5 °C (Fig. 3B). For example, though 696 SGR was approximately the same and thus the fraction of biomass replaced per time similar 697 across all experimental temperatures (0.147 and 0.141 at 13 °C and 26.5 °C, respectively; Fig. 698 3B) microbes at 13 °C generated 20.9 mg biomass h⁻¹ while those at 26.5 °C generated less than 699 half the amount (9.5 mg biomass h^{-1}). – 700
- 701 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 <u>steady-state</u> 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 <u>for soils and aquatic ecosystems</u> (del Giorgio and Cole, 1998;
Gillooly et al., 2001; <u>Rivkin and Legendre, 2001;</u> 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 709 pronounced differences in δ^{13} C between microbial biomass and the sole C substrate, cellobiose, 710 711 and between microbial biomass and respired CO_2 (Fig. 4). Microbial biomass exhibited 5.5 to 10.5% more negative δ^{13} C values than the cellobiose and respired CO₂ was even more 13 C 712 depleted, at least 4.4‰ more negative than the biomass (Fig. 4A). Because each chemostat was 713 at steady-state, isotopic mass balance dictates that ¹³C "missing" from cellobiose had to 714 715 accumulate in another pool in the reactor. The only pool that could have been enriched with the "missing" ¹³C was reactor DOC, which we analyzed for δ^{13} C in four out of the seven chemostat 716 runs (Fig. 4A). Reactor DOC consisted of a large pool of cellobiose (because the rate of C 717 718 consumption by the chemostat cultures was, on average, only 42.8% of the rate of C supply) and presumably a pool of additional organic compounds (e.g. such as acetate). Such compounds 719 appear to be typically secreted from microbial cells at low rates in aerobic chemostats operated at 720 dilution rates similar to those of our runs (El-Mansi and Holms, 1989; Nanchen et al., 2006), and 721 have been shown to be enriched in ¹³C compared to cellular biomass (Blair et al., 1985). 722 However, because such a small fraction of the available cellobiose was taken up by P. 723 fluorescens, the fraction of total DOC comprised of secreted organic compounds was small. As a 724

result, ¹³C 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 727 likely due to discrimination against 13 C during cellobiose uptake. If we assume that P. 728 729 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 ¹³C-containing cellobiose 730 during uptake (and thus assimilated cellobiose possessed a δ^{13} C of -24.2%), isotopic mass 731 balance dictates that the δ^{13} C of the C secretion flux (Fig. 5) would have to be +70‰, at 732 minimum, across all temperatures. To our knowledge, such high metabolic discrimination 733 against ¹³C would be very unusual for biological systems (O'Leary, 1981). An alternative and 734 more likely scenario is therefore that *P. fluorescens* took up less ¹³C-containing cellobiose than 735 was supplied as substrate, and that discrimination during uptake contributed substantially to P. 736 *fluorescens* biomass and respired CO_2 being more ¹³C depleted than the cellobiose supplied. 737 This conclusion holds for all temperatures studied. If we assume for the example 23.5 °C 738 chemostat run at steady-state (Figs. 2, 5) that *P. fluorescens* secreted organic compounds at a rate 739 of 10% of the sum of SGR and SRR, and we further assume that the δ^{13} C of secreted compounds 740 was 11.7% less negative than that of the biomass (Blair et al., 1985), that is -18.1%, then did not 741 discriminate against 13 C during secretion but only during uptake, the δ^{13} C of the cellobiose taken 742 up would have been, on average, -34.2-31.1%, which is only a 10% difference from the 743 cellobiose provided, and therefore probably a more likely scenario (Fig. 5). 744 Substantial ¹³C depletion of respired CO₂ relative to microbial biomass has not, to our 745

knowledge, been reported in other studies. With the microbial C consumption rate amounting to

only 1.82.8% of the rate of C supply, C availability was high compared to what soil microbes in 747 their natural environments typically experience (Tempest and Neijssel, 1978; Cole et al., 1988; 748 Hobbie and Hobbie, 2013), potentially promoting enzymatic discrimination. The cellobiose δ^{13} C 749 of -24.2 % implies a ¹³C/¹²C ratio of ~1/91. Considering the molecular formula of cellobiose 750 $C_{12}H_{22}O_{11}$, this means that not more than about one out of eight cellobiose molecules in the 751 supplied substrate included a ¹³C atom. Faster diffusion of the isotopically lighter cellobiose 752 molecules may have contributed to a lower probability of ¹³C-containing cellobiose approaching 753 bacterial membrane uptake sites, and hence, to the differences between $\delta^{13}C$ of substrate and 754 biomass (Fig. 4A). However, isotope fractionation during diffusion – a physical process 755 dependent on compound mass – would likely exhibit a continuous temperature response. Thus, 756 it seems unlikely that fractionation during diffusion was the primary driver of the pronounced, 757 discontinuous changes in the difference between substrate and biomass δ^{13} C, which ranged from 758 759 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 760 761 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 762 was growing and the related differences in substrate uptake rates. 763

764Evidence from work on C isotope distribution within carbohydrate molecules (e.g., Rossmann et

765 <u>al., 1991; Gleixner and Schmidt, 1997) suggests non-random distribution of ¹³C in biological</u>

766 molecules (such as cellobiose). Based on such phenomena, it is probable that the ¹³C atom in

767 $\frac{^{13}\text{C-containing cellobiose was consistently at the same position within the molecule, which rules}{^{13}\text{C-containing cellobiose was consistently at the same position within the molecule, which rules}$

- 768 <u>out the possibility that any changes in the intramolecular ¹³C distribution of cellobiose were</u>
- 769 responsible for the observed δ^{13} C patterns in biomass and respired CO₂ (Fig. 4). Hence, \mp the

discontinuous pattern of δ^{13} C of respired CO₂ with temperature, similar to the pattern for δ^{13} C of 770 the biomass (Fig. 4A), presumably reflects the downstream consequence of an upstream change 771 in δ^{13} C of the metabolic substrate taken up and ultimately respired. However, the more negative 772 δ^{13} C of respired CO₂ compared to that of biomass is, to our knowledge, the most direct evidence 773 to date for ¹³C discrimination during respiration of a heterotrophic soil-microbe. The observation 774 of a substantial respiratory ¹³C discrimination corroborates inferences drawn in earlier studies 775 776 (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; 777 Bathellier et al., 2009; Ghashghaie and Badeck, 2014). Our observations of respiratory 778 discrimination against ¹³C highlight the similarity of heterotrophic, aerobic respiratory pathways, 779 and isotope effects within them, across life's domains. 780

In contrast to the discontinuous relationship between biomass δ^{13} C and temperature, we observed 781 a comparably continuous and linear increase in respiratory discrimination against ¹³C with 782 temperature (Fig. 4B). This increase generated a marginally positive significant (P=0.08) 783 784 correlation with SRR (Fig. 6), and hence a marginally significant (P=0.07) correlation with CUE. 785 A physiological interpretation of this finding is not straightforward, as multiple, possibly simultaneous enzymatic fractionations may have contributed to the observed δ^{13} C of respired 786 CO₂ (Dijkstra et al., 2011; Tcherkez et al., 2012). It could simply result from a proportionally 787 increasing flux through respiratory pathways, with associated stronger expression of 13 C 788 discrimination by the enzymes involved (Tcherkez et al., 2012), or could result from increasing 789 temperatures altering the relative fluxes through respiratory pathways (Chung et al., 1976; 790 Wittmann et al., 2007; Dijkstra et al., 2011) such that the overall observed respiratory ¹³C 791 discrimination increased with temperature. This may be possible given that respiratory pathways 792

793 can exhibit distinct fractionation factors (Bathellier et al., 2009 and references therein) and prompt different, specific C atoms to undergo decarboxylation from the two glucose units of the 794 substrate cellobiose, which contain non-randomly distributed ¹³C atoms (Rossmann et al., 1991; 795 796 Gleixner and Schmidt, 1997). If relative fluxes through different respiratory pathways changed with temperature, the continuous nature of the relationship between temperature and respiratory 797 ¹³C discrimination suggests a smooth transition compared to the abrupt and discontinuous shifts 798 799 in apparent uptake and/or secretion discrimination described above. Future metabolic flux analyses linked to isotopic approaches sensitive enough to quantify C isotopes in microbial 800 801 exudation will be well-suited to explore how C allocation to distinct, aerobic respiratory pathways may vary with temperature and result in varying δ^{13} C of respired CO₂. 802

803

4. Conclusions

Our observations clearly show a decline in microbial CUE with increasing temperature when C 805 806 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, temperature dependent SRR, which influences CUE and growth efficiency, in ecosystem process 808 models. The temperature-driven changes in SRR and respiratory discrimination against ¹³C were 809 not independent of each other, suggesting that increasing SRR, to some degree, drives enhanced 810 C isotopic discrimination. We demonstrate that C isotope discrimination associated with 811 microbial decomposition of SOM can impart large and variable isotopic signatures on C pools 812 typically characterized and interpreted in biogeochemical studies at any scale. To date, efforts to 813 partition flux components of net ecosystem exchange have assumed little to no fractionation 814

815	between respired substrates and the resultant CO ₂ . Our results suggest that this assumption must
816	be reevaluated, and represent a first step towards an isotopically explicit, mechanistic framework
817	for microbial C isotope fluxes in Earth system models.
818	
819	Author contribution:
820	C.A.L. and K.M. performed the experiments; all authors contributed to all other parts and stages
821	of the manuscript.
822	
823	Data availability: The data presented in this study are available for collaborative use by anyone
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1008 Figure legends:

Fig. 1. Chemostat system comprised of *P. fluorescens* growing on cellobiose. Seven independent 1009 experiments were conducted, with reactor temperatures of 13, 14.5, 16, 18, 21, 23.5 and 26.5 °C; 1010 all other conditions were identical. During continuous flow, dilution rate of the reactor 1011 (mean= 0.137 ± 0.01 h⁻¹ across all experiments) equals microbial growth rate. A peristaltic pump 1012 1013 supplied fresh nutrient medium from a reservoir tank to the reactor and removed reactor medium 1014 (including biomass) at a constant rate. Headspace volume was flushed with CO₂-free air, bubbling through reactor medium and supplying microorganisms with O_2 . A ${}^{13}CO_2/{}^{12}CO_2$ 1015 analyzer continuously sampled reactor headspace and measured the concentration and δ^{13} C of 1016 respired CO_2 . 1017

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Fig. 2. Example time course of the evolution of reactor headspace CO₂ concentration (A) and δ^{13} C of the CO₂ (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 δ^{13} C of respired CO₂ were measured between 70 and 74 h after inoculation when the culture reached steady-state.



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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. 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.

