

Chemoautotrophic carbon fixation in thermokarst lakes on the Tibetan Plateau

Corresponding Author: Professor Yuanhe Yang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This study examined dark carbon fixation by chemoautotrophs in thermokarst lakes of the Tibetan Plateau. Large scale sampling across the Tibetan Plateau was conducted, along with ^{14}C -labeling incubation and molecular analysis. They found that dark carbon fixation exists in thermokarst lakes, and nitrifying microorganisms are the main drivers via Calvin-Benson-Bassham cycle. It is significant for evaluation of carbon cycling in thermokarst lakes, especially on the Tibetan Plateau. However, the following concerns of this study should be addressed before further consideration.

1. Dark carbon fixation can exist in the whole water column as well as the sediment, but only surface water samples were collected, which also leads to an incomplete and inaccurate understanding of permafrost C-climate feedback.
2. Only lakes exhibited DOC accumulation were selected to conduct ^{14}C -labeled incubation. However, DOC loss only demonstrate that DOC degradation exceeds C fixation, dark C fixation may also exist in DOC decreasing lakes. The ^{14}C -labeled incubation should also be conducted in representing DOC loss sites. It is also not appropriate to declare that one-third of the studied thermokarst lakes experience significant C fixation process dominated by chemoautotrophs in Lines 35-36.
3. C fixation can contribute to DOC accumulation based on Lines 129-132. But there were no data showing the percentage of dark C fixation contribution to the total DOC, which can demonstrate the importance of dark C fixation. The fixed C can be calculated via dark C fixation rates, and should be compared to the DOC increase.
4. Line 153-154, it is not solid evidence to confirm the absence of 3-HP/4-HB cycle using PCR electrophoresis assay. Metagenomics should be used as clear evidence.
5. On the other hand, it is not surprising to find CBB cycle act as the main pathway, as CBB cycle takes advantage in most aerobic waters.
6. Lines 172-178, this is only data correlation rather than mechanisms, which can't be solid evidence to demonstrate ccbL takes advantages.
7. Lines 221-226, since the amplicon sequencing was based on cbbL gene, the results only show that nitrifiers dominant in cbbL containing microbes. Sequencing based on other functional genes may have different results.
8. The rates of nitrification should be measured, as nitrifiers are claimed to take advantages. Also, nitrification rates should have significant correlation with dark C fixation to confirm nitrifiers really dominant chemoautotrophs.
9. The gene abundances are not always equal to the real activities of functional microbes. Thus, DNA-SIP or metatranscriptome should be conducted to further reveal the microbial mechanisms.

Reviewer #2

(Remarks to the Author)

Ms. Ref. No.: NCOMMS-24-01621-T

Title: Substantial chemoautotrophic carbon fixation in thermokarst lakes on the Tibetan Plateau

In this study, the authors investigated the potential for chemoautotrophic C fixation in thermokarst lakes. They combined biodegradable DOC experiments and ^{14}C -labeling bicarbonate assimilation incubations using water samples from 188 thermokarst lakes along a 1,100-km permafrost transect across the Tibetan Plateau. They found that 1/3 of the studied thermokarst lakes experienced significant C fixation dominated by chemoautotrophs resulting in DOC accumulation rather

than loss during the incubation period. They also found that C fixation is driven primarily by nitrifying microbes using the *cbbL* gene and corresponding Calvin-Benson-Bassham cycle. They propose that chemoautotrophic C fixation predominates in some thermokarst lakes, partially offsetting substantial C emissions caused by permafrost thaw and weakening of the permafrost C-climate feedback.

Overall, this is an interesting manuscript focused on chemoautotrophic C fixation in thermokarst lakes, which has been largely ignored in the literature. However, while the authors' use of incubation experiments and quantitative PCR is commendable, I believe that their experimental design and broad interpretations requires additional validation before I can recommend this paper for publication.

Noteworthy Results:

This study was observational by design, yet its experimental results highlight the potential for chemoautotrophic C fixation in a subset of thermokarst lakes across the Tibetan Plateau. There are two particularly noteworthy results, (1) identifying that thermokarst lakes in swamp meadow have higher C fixation rates than lakes in alpine steppe or alpine meadows, and (2) DOC fixation was driven primarily by nitrification with nitrifying bacteria being the dominant group of chemoautotrophs in these lakes.

Validity:

The authors' primary conclusion that chemoautotrophic C fixation is the predominant microbial process that occurs in a subset of thermokarst lakes can only be partially validated by their experimental design and subsequent results. It is important to note that their methodological approach limits their primary conclusion as well as their broader conclusions regarding the partial offsetting of substantial C emissions caused by permafrost thaw. Specifically, their incubation results are presented in a binary fashion such that an individual thermokarst lake either degrades DOC or fixes DOC. They conclude that 1/3 of sampled lakes fix DOC based on a single sampling time point using 100 mL of filtered surface lake water in a 28-day dark incubation experiment. The reality is much more nuanced, where there is likely DOC degradation and fixation occurring simultaneously within each lake that varies seasonally and by depth based on the changing composition of the microbial community and abiotic factors. Their experimental design cannot capture these nuances and their conclusions are too simplified to be applied broadly across the sampling region. At best, their results can only suggest that chemoautotrophic C fixation is one of potentially many possible microbial processes affecting DOC concentrations in thermokarst lakes.

Their second conclusion that chemoautotrophic C fixation occurs predominately through nitrification using the *cbbL* gene and Calvin-Benson-Bassham cycle can also be only partially validated by their methodological approach. The use of specific primers for genes encoding chemoautotrophic fixation pathways (3-HP/4-HB and CBB cycles) rather than a metagenomic approach that captures the full potential of genes available to the community limits the full scope of their analysis. The authors have done a fine job linking the abundance of *cbbL* genes with nitrifying bacteria in their incubations by drawing on previous literature, but without metagenomic insights, they may be missing other important chemoautotrophic components in the community that their PCR-based approach cannot capture.

Significance:

The significance of this research is the discovery of substantial potential for chemoautotrophic C fixation by nitrifying bacteria in thermokarst lakes. As the authors mention, chemoautotrophic fixation of DOC has been historically understudied relative to DOC degradation processes following permafrost thaw. This manuscript is a great first step providing proof of concept for potential chemoautotrophic fixation activity and the authors did well to determine a potential genetic mechanism for chemoautotrophy through the increased abundance of nitrifying functional genes (*cbbL*) and associated bacterial taxa. However, the lack of repeated measures within each lake or across seasons (and years) limits the validation of this response to a one-off observation within each individual lake across the sampling gradient. The methodological lack of validation and the binary nature of their analysis limits the interpretation of these results and the authors' conclusion that chemoautotrophic C fixation could offset substantial C emissions caused by permafrost thaw. These limitations on the interpretation and scalability of the results weakens the broader significance of this research and whether it merits publication in Nature Communications.

Data and Methodology:

I have several concerns regarding the methodology. My first concern is that the authors limited their radiolabeled bicarbonate incubations and all microbial analyses to the 40 randomly selected lakes that accumulated DOC in their biodegradable DOC experiment. I understand that costs were an issue, but it would have been nice to see results from at least one and preferably several lakes where DOC concentrations declined, to serve as a control. What is the microbial composition of those 119 lakes where DOC degradation increased? Are there chemoautotrophic taxa in those lakes? Can we use microbial 16S sequencing as a screening mechanism for determining if a thermokarst lake has the potential to fix DOC through chemoautotrophic pathways? The lack of data from 2/3 of the lakes makes it difficult to validate the significance of the results presented in the manuscript.

My second concern is the lack of gas measurements (CO₂) from the incubation experiments. If their broader claim is that chemoautotrophy will fix DOC rather than increase CO₂ emissions through DOC degradation processes, then it seems reasonable that there should have been measurements of CO₂ production from thermokarst lakes where DOC was degraded versus those lakes where DOC was fixed. It is difficult to determine if chemoautotrophic fixation can offset C emissions if there is no experimental validation.

Concluding Thoughts:

The authors' efforts to discover and quantify the extent of chemoautotrophic C fixation activity and the microbial mechanisms likely driving this metabolic process in thermokarst lakes is commendable. These results clearly show that this process should be considered as an important microbial factor when assessing the fate of DOC following permafrost thaw. However, this study serves best as an initial proof of concept for chemoautotrophic C fixation by nitrifying taxa. The over-simplification of the results (binary response) and lack of additional sampling time points limits the validation of the primary and broader conclusions as presented. The paper is technically sound regarding the discovery of chemoautotrophic C fixation but remains too speculative to support the broader significance of the results regarding the potential for chemoautotrophic C fixation to partially offset substantial C emissions caused by permafrost thaw and weakening of the permafrost C-climate feedback. The lack of supporting gas measurements is just one of several limiting factors that make the conclusions of this study too speculative as written.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Reviewer #2

(Remarks to the Author)

I would like to commend the authors for their exceptionally thorough and thoughtful revision. It is clear that a great deal of additional work—both in the field and in the laboratory—was undertaken to address the comments raised in the initial review. The inclusion of multi-season sampling across the water column, CO₂ gas measurements, ¹⁴C-labeled incubations for DOC-decreasing lakes, and metagenomic analyses substantially strengthens the manuscript and provides robust validation of the original conclusions.

Specifically:

- The addition of seasonal and depth-resolved sampling convincingly resolves the concern regarding the single-time-point limitation and provides valuable insights into the temporal and spatial variability of chemoautotrophic C fixation.
- The incorporation of CO₂ flux measurements offers critical experimental validation for the proposed offsetting of C emissions and demonstrates methodological rigor.
- The metagenomic analyses are particularly commendable, as they clarify the dominance of the CBB cycle and the role of the *cbbL* gene in dark C fixation, thereby addressing the previous limitation of relying solely on targeted PCR assays.
- Finally, the inclusion of 16S rRNA and functional gene analyses for both DOC-increasing and DOC-decreasing lakes offers a balanced view of microbial community structure and function across contrasting lake states.

Overall, this revised manuscript represents a substantial improvement over the original version and now provides strong, well-supported evidence for chemoautotrophic carbon fixation in thermokarst lakes. The authors' commitment to addressing the earlier critiques through additional field sampling, gas flux measurements, and metagenomic analyses is commendable and elevates the scientific rigor and impact of the work.

I appreciate the authors' thoughtful revisions and am pleased to recommend the manuscript for publication in Nature Communications.

Sincerely,
Karl J. Romanowicz, Ph.D.
Department of Bioengineering
University of Oregon

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Responses to Reviewer #1

[Comment 1] This study examined dark carbon fixation by chemoautotrophs in thermokarst lakes of the Tibetan Plateau. Large scale sampling across the Tibetan Plateau was conducted, along with ^{14}C -labeling incubation and molecular analysis. They found that dark carbon fixation exists in thermokarst lakes, and nitrifying microorganisms are the main drivers via Calvin-Benson-Bassham cycle. It is significant for evaluation of carbon cycling in thermokarst lakes, especially on the Tibetan Plateau. However, the following concerns of this study should be addressed before further consideration.

[Response] Thanks for the reviewer's insightful comments. The comments listed below enabled us to have a deeper thinking on this issue, and thus guided us to conduct a thorough revision of the original manuscript (MS). We have carefully addressed your comments through the following major changes:

- We re-collected water samples in the whole water column and sediments of thermokarst lakes during 2024, which were then used to explore the dissolved organic carbon (DOC) dynamics so as to address the limitation of method only based on surface water sampling in our initial research.
- We added ^{14}C -labelled incubation to explore whether the dark carbon (C) fixation occurred in thermokarst lakes with DOC loss.
- We performed metagenomic sequencing to demonstrate that the dark C fixation was processed mainly through the CBB cycle rather than other chemoautotrophic C fixation pathways such as 3-HP/4-HB.
- We determined the nitrification rates of thermokarst lakes based on ^{15}N -isotope incubation experiment, and illustrated that the dark C fixation was primarily fueled by nitrification via nitrifiers.

Addressing these points has significantly strengthened the quality of our MS. Please see our responses to the comments listed below for detailed modifications.

[Comment 2] Dark carbon fixation can exist in the whole water column as well as the

sediment, but only surface water samples were collected, which also leads to an incomplete and inaccurate understanding of permafrost C-climate feedback.

[Response] We agree with the reviewer's point that "*dark carbon fixation can exist in the whole water column as well as the sediment*". To verify this point, **we re-collected water samples in the whole water column from thermokarst lakes in 2024**, where **the surface sediments** (0-1 cm; surface sediments are the area with the most active metabolism of chemoautotrophs which could represent the *in-situ* dark C fixation) were also sampled in the corresponding lakes (Santoro et al., 2013; Dyksma et al., 2016). Based on these newly collected samples, we conducted the bioincubation experiment and radiolabeled bicarbonate ($\text{NaH}^{14}\text{CO}_3$) incubation to determine the C dynamics in water and corresponding sediments of investigated thermokarst lakes.

For water samples, we employed three kinds of methods to verify whether dark C fixation existed in these lakes. First, based on the bioincubation experiment by determining the variations of DOC concentration (Vonk et al., 2015), we found that **30.8% of lakes increased organic C in surface layer (0-20 cm), and 36.0% of lakes accumulated DOC in sublayer** (> 20 cm; Fig. R1a-b). Second, to confirm the reliability of bioincubation experiment mentioned above and respond to the comments from **Reviewer #2**, we further conducted another bioincubation experiment based on CO_2 measurement (Wickland et al., 2012), and found similar results to those determined based on the variations of DOC concentration (Fig. R1c-d). Third, we **performed ^{14}C -labelling incubation for water samples and** observed that dark C fixation rates ranged from 0.04 to 2.05 $\mu\text{g C L}^{-1} \text{ d}^{-1}$ in the surface layer while those ranged from 0.09 to 1.11 $\mu\text{g C L}^{-1} \text{ d}^{-1}$ in the sublayer (Fig. R1e-f). **These three lines of evidence proved the existence of dark C fixation in the whole water column,** and also demonstrated that partial thermokarst lakes experienced the process that dark C fixation exceeded DOC degradation, resulting in DOC accumulation rather than loss.

For the sediments, we conducted ^{14}C -labelling incubation and found that chemoautotrophic C fixation also existed in these lake sediments, with rates ranging from 3.42 to 25.70 $\text{mg m}^{-2} \text{d}^{-1}$ (Fig. R2). Moreover, the dark C fixation rates of sediments in thermokarst lakes were positively associated with those in water samples. That is, when thermokarst lakes had relatively higher dark C fixation rates in water bodies, they also had greater rates of C fixation in corresponding sediments. Taken together, these results determined from newly collected samples revealed that chemoautotrophic C fixation existed in the whole water column and lake sediments. We have clearly stated this point and added the related descriptions involving the method, results and discussion to the revised MS (Pages 5-7, lines 110-140; Pages 15-16, lines 328-343; Pages 17-21, lines 360-457).

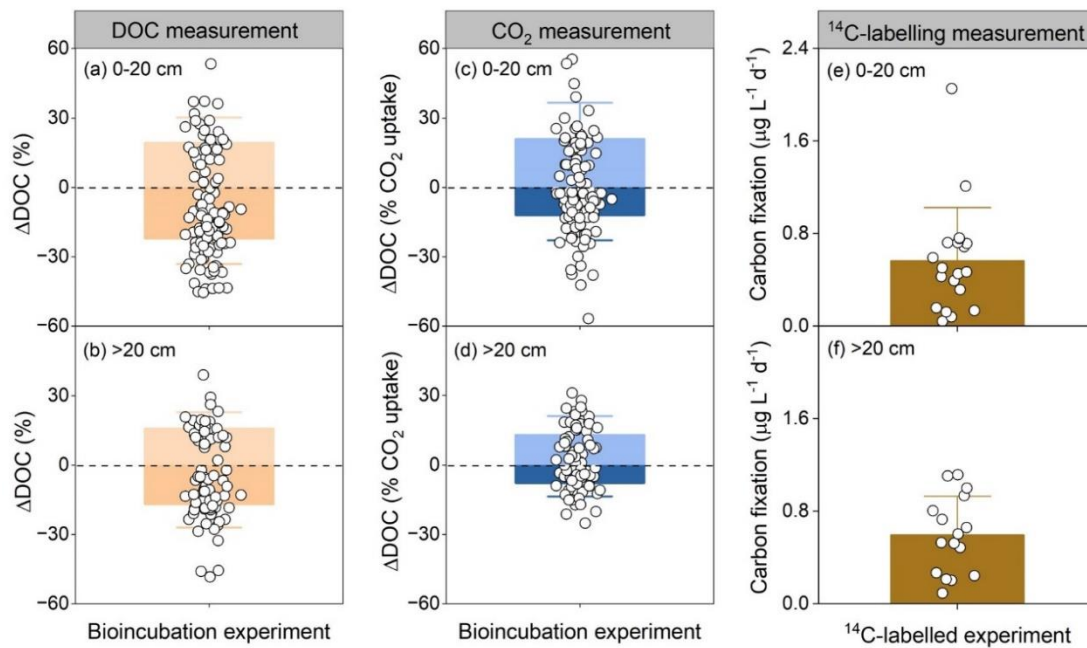


Fig. R1. Changes in DOC of thermokarst lakes during the incubation and their dark C fixation rates. **a, b**, The changed percentage of DOC in the surface (a) and subsurface water (b) of thermokarst lakes during a 28-day incubation determined based on DOC concentration variations (Vonk et al., 2015). **c, d**, The changed proportion of DOC in the upper (c) and lower water (d) of thermokarst lakes based on CO_2 measurement (Wickland et al., 2012). **e, f**, Dark C fixation rates in surface (e) and subsurface water (f) of thermokarst lakes on the basis of ^{14}C -labelling incubation (Berg

et al., 2015). If ΔDOC was positive, it represented the increased proportion of DOC during the incubation. By contrast, if ΔDOC was negative, it indicated the proportion of DOC loss during the incubation. The circles distributed on the bar indicate the data of examined water samples in each thermokarst lakes. Notably, considering that the depth of ~85% of thermokarst lakes was below 50 cm (Supplementary Table 3 in the revise MS), we mainly divided the investigated lakes into surface layer (0-20 cm) and sublayer (> 20 cm) for data analysis.

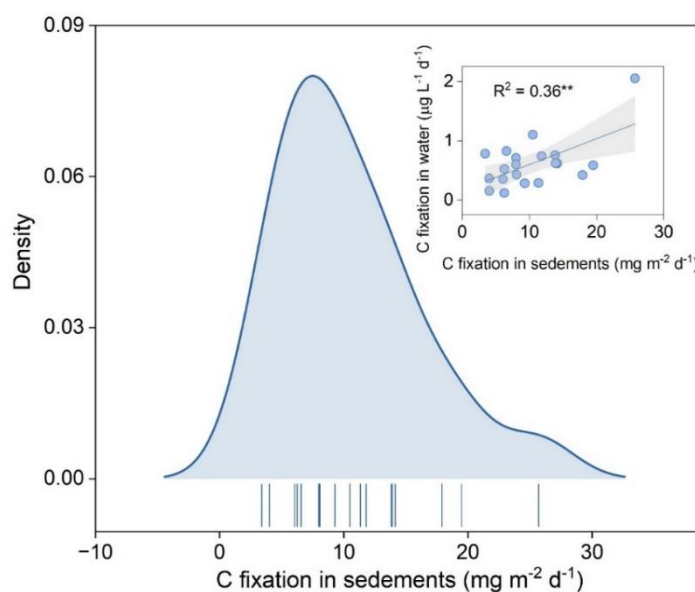


Fig. R2. Dark C fixation rates of surface sediments from the investigated thermokarst lakes Inset shows the relationship between C fixation rates in water and those in sediments of thermokarst lakes collected in summer of 2024. Dark C fixation rates in water and sediments were determined based on ^{14}C -labelling incubation (Santoro et al., 2013; Berg et al., 2015). The solid lines denote the fitted ordinary least-squares model, and the gray areas indicate the 95% confidence intervals. ** $P < 0.01$.

[Comment 3] Only lakes exhibited DOC accumulation were selected to conduct ^{14}C -labeled incubation. However, DOC loss only demonstrate that DOC degradation exceeds C fixation, dark C fixation may also exist in DOC decreasing lakes. The ^{14}C -labeled incubation should also be conducted in representing DOC loss sites. It is also not appropriate to declare that one-third of the studied thermokarst lakes experience significant C fixation process dominated by chemoautotrophs in Lines 35-36.

[Response] Very good comments! Following the reviewer's comments, **we conducted ^{14}C -labelling incubation for the lakes that suffered DOC loss.** Our results **illustrated that the dark C fixation process also existed in DOC decreasing lakes, with rates ranging from 0.04 to 0.76 $\mu\text{g C L}^{-1} \text{d}^{-1}$ with a mean of $0.44 \pm 0.05 \mu\text{g C L}^{-1} \text{d}^{-1}$** (Fig. R3a). In the remaining thermokarst lakes with the occurrence of DOC accumulation, the C fixation rates varied from 0.10 to 2.05 $\mu\text{g C L}^{-1} \text{d}^{-1}$, with a mean of $0.74 \pm 0.12 \mu\text{g C L}^{-1} \text{d}^{-1}$ (Fig. R3b).

Interestingly, the C fixation rates of thermokarst lakes occurring with DOC accumulation was significantly higher than those occurring with DOC degradation (Fig. R3c). In the lakes occurring with DOC loss, we found no significant correlation between the dark C fixation rates and the decreased percentage of DOC during the incubation period (Fig. R3a). By contrast, in the lakes exhibiting DOC accumulation, the increased percentage of DOC was positively associated with the rates of dark C fixation (Fig. R3b). These results indicated that the dark C fixation had limited effects on the DOC dynamics of lakes occurring with DOC loss, but could exert crucial roles in mediating DOC dynamics in the DOC increasing lakes. Such a phenomenon also implied that the DOC decreasing lakes experienced the process that DOC degradation exceeded dark C fixation, finally resulting in DOC loss. While in the DOC increasing lakes, they suffered the process that dark C fixation exceeded DOC degradation, leading to DOC accumulation. We have clearly stated this point in the revised MS (Pages 6-7, lines 121-140).

Based on new experiments mentioned above, we agree with the reviewer's point that "dark C fixation may also exist in DOC decreasing lakes" and **have re-organized this sentence in the revised MS as follows:** "*We observe that dark C fixation exceeds DOC degradation in $\sim 1/3$ of the investigated thermokarst lakes across the Tibetan Plateau, resulting in DOC accumulation rather than loss during the incubation period*" (Page 2, line 36-39).

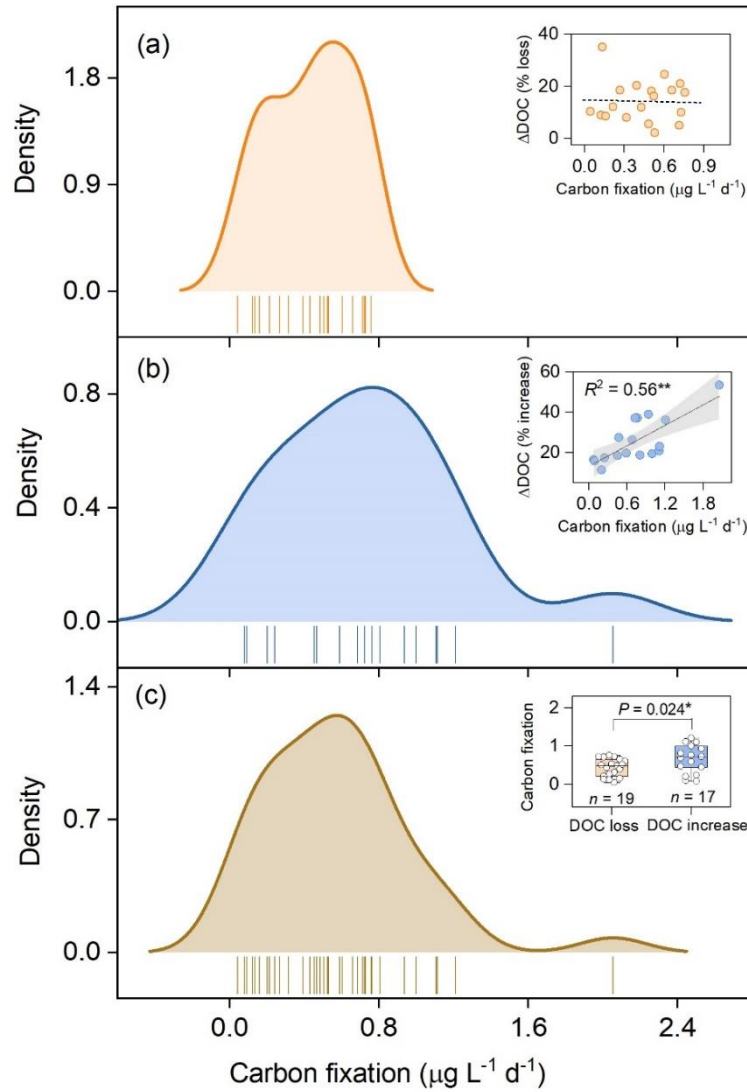


Fig. R3. Dark C fixation rates from the investigated thermokarst lakes collected in summer of 2024. **a, b,** Dark C fixation rates from the thermokarst lakes occurring with DOC degradation (a) and DOC accumulation (b). Inserts in panels (a) and (b) shows the relationships of dark C fixation rates with ΔDOC (% loss) and ΔDOC (% increase) in thermokarst lakes, respectively. The solid lines denote the fitted ordinary least-squares model, and the gray areas indicate the 95% confidence intervals. **c,** Dark C fixation rates in total water samples including those occurring with DOC degradation and accumulation. Inset in panel (c) shows the comparisons of dark C fixation rates among all the thermokarst lakes occurring with DOC loss ($n = 19$) and accumulation ($n = 17$). Notably, considering the high experimental cost, we randomly selected 20 of the investigated thermokarst lakes with a total of 36 water samples being collected, and

then calculated the dark C fixation rates for each sample. Dark C fixation rates were determined on the basis of ^{14}C -labelling incubation (Berg et al., 2015). ΔDOC (% increase) and ΔDOC (% loss) denote the increased and decreased percentage of DOC during incubation determined based on DOC measurement (Vonk et al., 2015), respectively. * $P < 0.05$ and ** $P < 0.01$.

[Comment 4] C fixation can contribute to DOC accumulation based on Lines 129-132. But there were no data showing the percentage of dark C fixation contribution to the total DOC, which can demonstrate the importance of dark C fixation. The fixed C can be calculated via dark C fixation rates, and should be compared to the DOC increase.

[Response] Following the reviewer's comments, we calculated the percentage of dark C fixation (determined by ^{14}C -labelled incubation) contribution to DOC accumulation (examined by bioincubation experiment), which ranged from 0.4% to 7.9% (Fig. R4a).

The low contribution of dark C fixation to DOC accumulation could be largely ascribed to different analytical principles of the two methods. Specifically, for the bioincubation experiment, this method calculated the net DOC accumulation in water by determining DOC concentration during incubation (Vonk et al., 2015). By contrast, for the ^{14}C -labelled incubation, it examined chemoautotrophic C fixation rate by analysing the radioactive characteristics (disintegrations per minute) in bodies of chemoautotrophic communities (Berg et al., 2015). The difference in experimental principle of these two methods may lead to incomparable data. In addition, the cultivation time of DOC bioincubation is 28 days, while the ^{14}C -labelled incubation only needs two days. Overall, due to the differences in experimental principle and incubation time, these two methods are not directly comparable. The detailed descriptions about these two methods have been added to the revised MS (Pages 17-18, lines 360-385; Pages 19-20, lines 411-439).

Nevertheless, there was a positive correlation between the dark C fixation rates and the increased percentages of DOC during 28-day incubation (Fig. R4b),

suggesting that both methods could characterize large-scale patterns of dark C fixation across the thermokarst lakes. Moreover, although the contribution of dark C fixation was relatively low, we found that **the dark C assimilation rates in thermokarst lakes were similar to those in freshwater from other regions** (Noguerola et al., 2015; Vick-Majors and Priscu, 2019; [Table R1](#)), indicating that our data determined by ^{14}C -labelled incubation were reasonable. Thanks for your understanding!

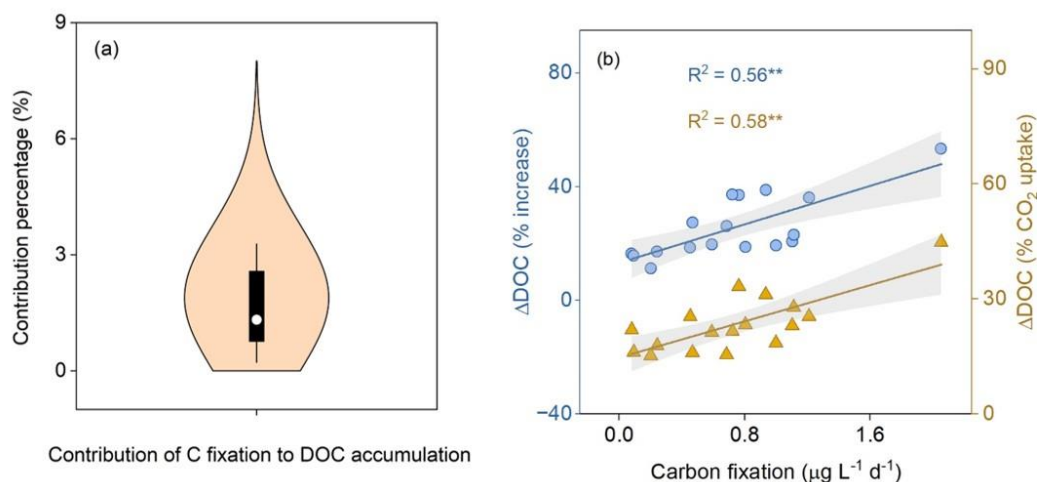


Fig. R4. Contribution of dark C fixation to DOC accumulation and the relationships of dark C fixation rates with ΔDOC (% increase) and ΔDOC (% CO_2 uptake). The whiskers within violin plot denote the 5th and 95th percentiles, and the ends of the boxes represent the 25th and 75th quartiles. The circle in the box represents the mean value of the data ($n = 17$). Notably, although dark C fixation rates were determined for all of 36 water samples from 20 selected thermokarst lakes, only 17 of the samples occurred with DOC accumulation that could be used for calculating the contribution of dark C fixation to DOC accumulation, while the remaining 19 samples suffered the process of DOC loss and cannot be used to calculate this contribution. Dark C fixation rates of thermokarst lakes were determined on the basis of ^{14}C -labelling incubation (Berg et al., 2015). ΔDOC (% increase) and ΔDOC (% CO_2 uptake) denote the increased percentage of DOC during incubation determined based on DOC (Vonk et al., 2015) and CO_2 measurements (Wickland et al., 2012), respectively. The solid lines denote the fitted ordinary least-squares model, and the gray areas indicate the 95% confidence intervals. **** $P < 0.01$.**

Table R1. The rates of dark C fixation in marine water, groundwater and lakes based on ^{14}C -labelling incubation.

Type	Region	Site	Depth (m)	Dark C fixation ($\mu\text{g L}^{-1} \text{d}^{-1}$)	References
Marine water	Mediterranean Sea	Tyrrhenian area	200-3480	0.05-0.24	(Yakimov et al., 2011)
Marine water	South China Sea	Shelf and offshore waters near Hainan Island	0-1500	0.48-4.08	(Zhou et al., 2017)
Marine water	Black Sea	Hydrographic stations in the western basin	5-95	1.36-3.96	(Karl and Knauer, 1991)
Marine water	Baltic Sea	Oxic-anoxic interface of deep sea	85-227	8.4-16.8	(Labrenz et al., 2005)
Marine water	North Atlantic	Eastern, subtropical and western North Atlantic	100-4039	0.001-0.68	(Reinthal et al., 2010)
Marine water	North Pacific	Eastern tropical North Pacific	0-2000	0.24-2.4	(Podlaska et al., 2012)
Groundwater	Germany	Wells from the Hainich Critical Zone	5-90	0.043-0.23	(Overholt et al., 2022)
Groundwater	Sweden	Groundwater borehole from crystalline bedrock	830-1078	0.0095-0.056	(Pedersen and Ekendahl, 1992)
Lake	Antarctic	Ice-covered lakes in the McMurdo Dry Valleys	6-20	0.04-2.1	(Vick-Majors and Priscu, 2019)
Lake	Spain	Lake Banyoles	3-12	0.24-1.20	(Noguerola et al., 2015)
Lake	Italy	Maggiore Lake	350	0.19	(Callieri et al., 2014)
Lake	China	Thermokarst lakes on the Tibetan Plateau	0.12-1.11	0.04-2.05	This study

[Comment 5] Line 153-154, it is not solid evidence to confirm the absence of 3-HP/4-HB cycle using PCR electrophoresis assay. Metagenomics should be used as clear evidence. On the other hand, it is not surprising to find CBB cycle act as the main pathway, as CBB cycle takes advantage in most aerobic waters.

[Response] Following the reviewer's comments, we conducted metagenomic sequencing to elucidate whether 3-HP/4-HB cycles existed in these lakes. Our results revealed that the functional genes involved in the 3-HP/4-HB cycle were not detected in 92% of examined samples. Moreover, the abundance of functional genes involved in the 3-HP/4-HB cycle was less than 0.1, which was largely lower than that in the CBB cycle (Fig. R5). Additionally, the functional genes involved in the 3-HP/4-HB cycle had no significant correlation with either the dark C fixation rates or the increased percentage of DOC determined based on DOC and CO₂ measurements (Fig. R6). These results derived from metagenomic sequencing provided solid evidence that there may be little or even no chemoautotrophic C fixation process based on the 3-HP/4-HB cycle in these lakes.

We agree with the reviewer's point that it is not surprising to find CBB cycle act as the main pathway (Callieri et al., 2014; Noguerola et al., 2015; Vick-Majors and Priscu, 2019). Nevertheless, this study provided the first large-scale evidence that chemoautotrophic C fixation existed in thermokarst lakes (this point was also highlighted by the editor '*this study sheds needed light on overlooked processes affecting carbon cycling in thermokarst lakes*', and commended by the second reviewer '*this is an interesting manuscript focused on chemoautotrophic C fixation in thermokarst lakes, which has been largely ignored in the literature.*' 'This manuscript is a great first step providing proof of concept for potential chemoautotrophic fixation activity and the authors did well to determine a potential genetic mechanism for chemoautotrophy through the increased abundance of nitrifying functional genes (*cbbL*) and associated bacterial taxa'), and partial lakes occurred with the process that dark C fixation exceeded DOC degradation, resulting in DOC accumulation rather loss. In our

case, exploring the pathway of dark C fixation was only used to explain the underlying microbial mechanisms for the C dynamics of thermokarst lakes. We have added the related results and discussion involving the metagenomics to the revised MS (Pages 7-8, lines 142-169). Thanks for your understanding!

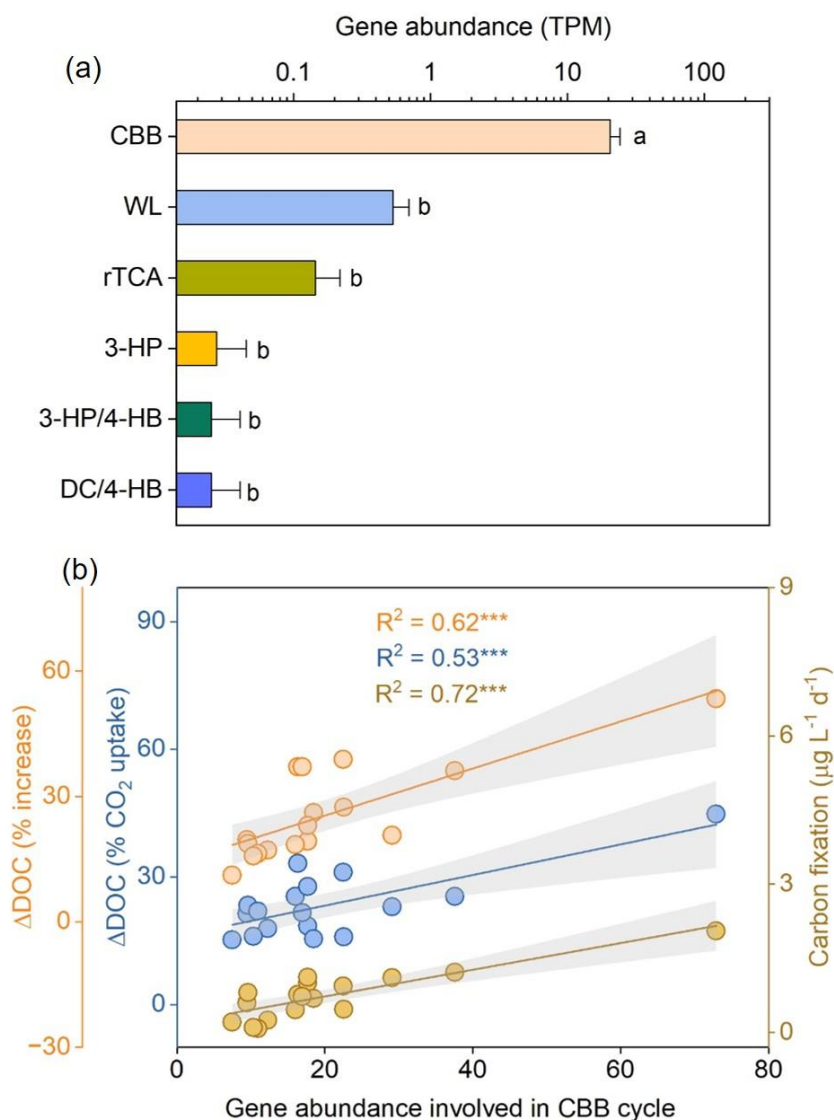


Fig. R5. Normalized abundances of functional genes involved in chemoautotrophic C fixation pathways and their associations with DOC accumulation and dark C fixation in thermokarst lakes during the incubation. **a**, Normalized abundances of genes involved in C fixation pathways of CBB, WL, rTCA, 3-HP, 3-HP/4-HB and DC/4-HB. **b**, Relationships of gene abundance in CBB cycle with Δ DOC (% increase), Δ DOC (% CO₂ uptake) and dark C fixation rates in thermokarst lakes. Δ DOC (% increase) and Δ DOC (% CO₂ uptake) denote the increased percentage of DOC during

the incubation determined based on DOC (Vonk et al., 2015) and CO₂ measurements (Wickland et al., 2012) for water samples collected in summer of 2024, respectively. Dark C fixation rates of thermokarst lakes were determined on the basis of ¹⁴C-labelling incubation for water samples collected in summer of 2024 (Berg et al., 2015). CBB, Calvin-Benson-Bassham cycle; WL, Wood-Ljungdahl pathway; rTCA, reverse glycine cleavage pathway; 3-HP, 3-hydroxypropionate bicycle; 3-HP/4-HB, 3-hydroxypropionate/4-hydroxybutyrate cycle; DC/4-HB, 4-hydroxybutyrate/dicarboxylic acid cycle. Different lowercase letters denote significant differences ($P < 0.05$) among the respective groups by one-way ANOVAs with LSD multiple comparisons. The solid lines denote the fitted ordinary least-squares model, and the gray areas indicate the 95% confidence intervals. Notably, statistically significant relationships of functional genes in CBB cycle with Δ DOC (% increase), Δ DOC (% CO₂ uptake) and dark C fixation in thermokarst lakes during the incubation remained stable even removing the rightmost point in panel (b). *** $P < 0.001$.

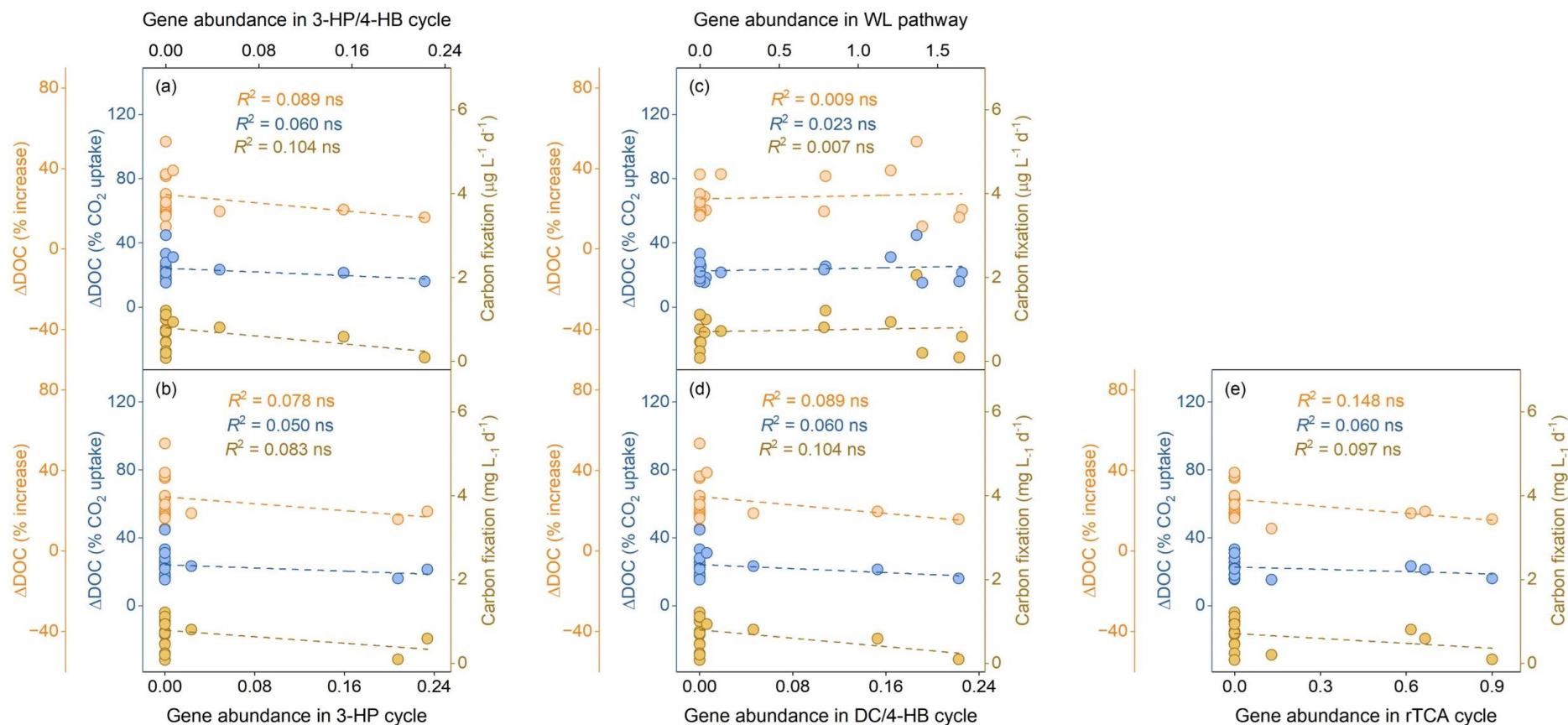


Fig. R6. Relationships of abundances of functional genes involved in chemoautotrophic C fixation pathways with the dark C fixation in thermokarst lakes. a-e, Relationships of genes involved in 3-HP/4-HB (a), 3-HP (b), WL (c), DC/4-HB (d), and rTCA (e) with ΔDOC (% increase), and ΔDOC (% CO_2 uptake) and dark C fixation rates in thermokarst lakes. ΔDOC (% increase) and ΔDOC (% CO_2 uptake) denote the increased

percentage of DOC during the incubation determined based on DOC (Vonk et al., 2015) and CO₂ measurements (Wickland et al., 2012) for water samples collected in summer of 2024, respectively. Dark C fixation rates of thermokarst lakes were determined on the basis of ¹⁴C-labelling incubation for water samples collected in summer of 2024 (Berg et al., 2015). 3-HP/4-HB, 3-hydroxypropionate/4-hydroxybutyrate cycle; 3-HP, 3-hydroxypropionate bicycle; WL, Wood-Ljungdahl pathway; DC/4-HB, 4-hydroxybutyrate/dicarboxylic acid cycle; rTCA, reverse glycine cleavage pathway. ns, $P > 0.05$.

[Comment 6] Lines 172-178, this is only data correlation rather than mechanisms, which can't be solid evidence to demonstrate cbbL takes advantages.

[Response] We agree with the reviewer's opinion that data correlation cannot be used as solid evidence to demonstrate cbbL takes advantages. To address the reviewer's concern, we provided two additional pieces of evidence to prove that cbbL rather than cbbM takes advantages in CBB cycle in thermokarst lakes. **First**, by conducting real-time fluorescence quantitative PCR, we found that the cbbL gene abundance was more than ten times higher than that of the cbbM gene (Fig. R7). **Second**, by performing metagenome-assembled genomes (MAGs), we obtained a total of 17 MAGs involved in the CBB cycle, of which 16 encoded form I Rubisco (cbbL), and one encoded form II Rubisco (cbbM) (Fig. R8). These two lines of evidence combined with the significant correlation between the cbbL gene copies and dark C fixation provided stronger evidence that chemoautotrophs in thermokarst lakes on the Tibetan Plateau mainly fixed C through the cbbL gene. We have clearly stated this point in the revised MS (Pages 8-9, lines 171-190).

Nevertheless, we acknowledge that statistical correlations do not necessarily reflect true causal relationships, and have mentioned this shortcoming in the Discussion section of the revised MS as follows: “*Second, despite that the abundance of functional genes (i.e., cbbL) in the CBB cycle was closely associated with chemoautotrophic C fixation, statistical correlations do not necessarily reflect causal mechanisms. Therefore, to elucidate the underlying mechanisms of chemoautotrophs more rigorously, future studies should incorporate RNA-based approaches (metatranscriptomics) and DNA-stable isotope probing (DNA-SIP) that systematically explore the active microbial processes underpinning dark C fixation in these environments, and further illustrate whether cbbL takes advantages in the CBB cycle within the studied thermokarst lakes*” (Pages 12-13, lines 265-272). Thanks for your understanding!

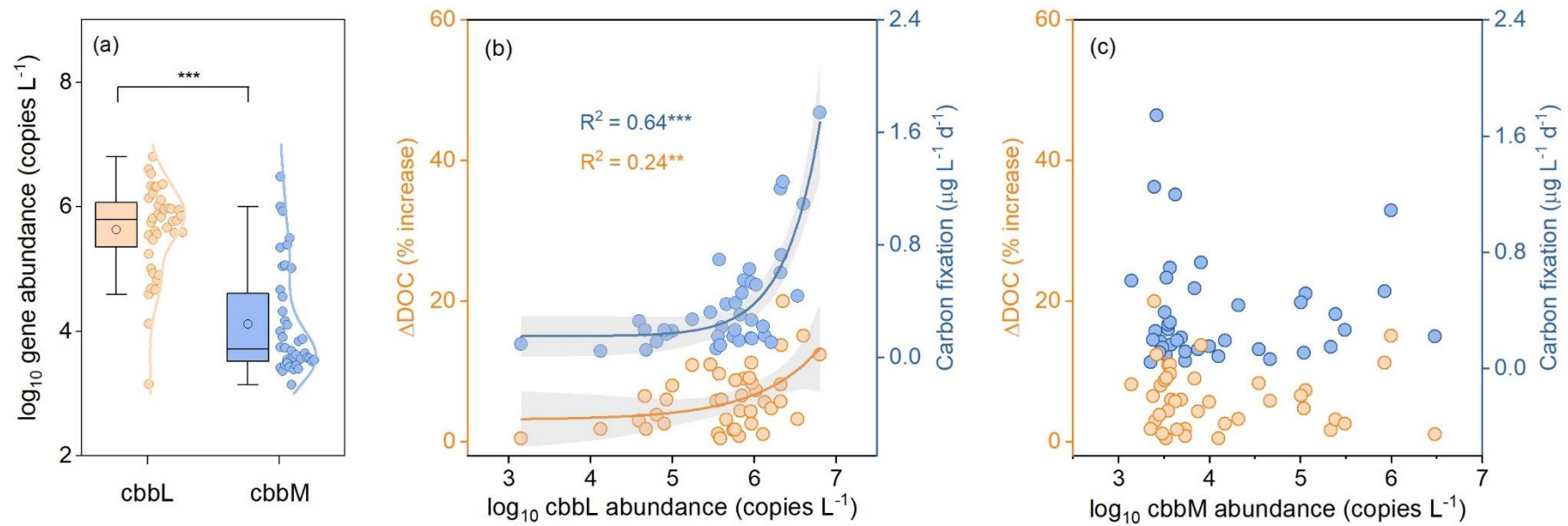


Fig. R7. Abundances of the cbbL and cbbM genes and their associations with dark C fixation. **a**, Differences in abundances between the cbbL and cbbM genes in thermokarst lakes where DOC accumulated. **b**, **c**, Relationships of the cbbL (**b**) and cbbM (**c**) genes with C fixation rates (orange line) and Δ DOC (% increase; blue line), respectively. Exponential fittings are used in panel (**b**) with the grey areas corresponding to 95% confidence intervals. Δ DOC (% increase) was examined by using a 28-day incubation determined based on DOC concentration measurement (Vonk et al., 2015) for water samples collected in summer of 2020. Dark C fixation was determined based on the $\text{NaH}^{14}\text{CO}_3$ assimilation experiment (Berg et al., 2015) for water samples collected in summer of 2020. Significant differences between the cbbL and cbbM gene abundances were determined based on a paired *t*-test. ** $P < 0.01$ and *** $P < 0.001$.

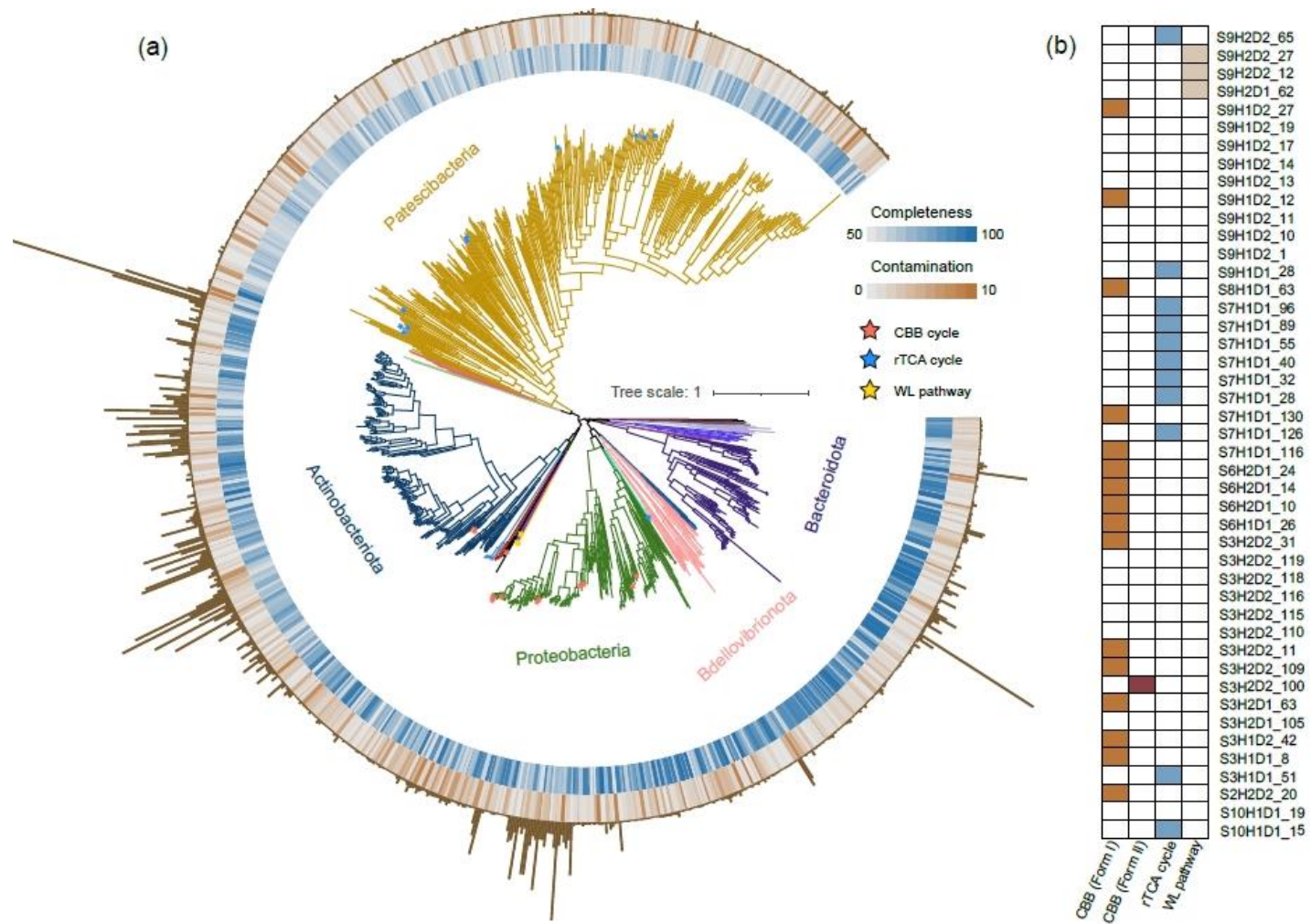


Fig. R8. Phylogeny and number of putative chemoautotrophic MAGs. **a**, Approximately maximum-likelihood phylogenetic tree was constructed based on single-copy concatenated protein using GTDB-Tk (Chaumeil et al., 2019). Colored pentagrams marked on the branch denote the predicted C fixation pathways, including CBB cycle (red), rTCA cycle (blue), and WL pathway (yellow). The first and second rings show the completeness and contamination of MAGs in this study. The outside bar of the second ring corresponds to the mean relative abundance of MAGs in thermokarst lakes collected in summer of 2024. **b**, Colored squares denote that MAGs for chemoautotrophic C fixation were detected in water samples. MAGs, Metagenome-assembled genomes; CBB, Calvin-Benson-Bassham cycle; rTCA, reverse glycine cleavage pathway; WL, Wood-Ljungdahl pathway.

[Comment 7] Lines 221-226, since the amplicon sequencing was based on cbbL gene, the results only show that nitrifiers dominant in cbbL containing microbes. Sequencing based on other functional genes may have different results.

[Response] As mentioned above in our response to *[Comments 5 and 6]*, based on metagenomic sequencing and real-time fluorescence quantitative PCR, we proved that chemoautotrophs in thermokarst lakes on the Tibetan Plateau mainly fixed C through the cbbL gene based on the CBB cycle. Due to this point, we analyzed the taxa encoding the cbbL gene using amplicon sequencing. By using phylogenetic tree analysis for the core operational taxonomic units (OTUs) with abundance >0.1%, we found that nitrifiers were dominated in cbbL containing microbes ([Fig. R9](#)). Therefore, the findings revealing that nitrifiers were the dominant group of chemoautotrophs in thermokarst lakes was reliable. We have clearly stated this point in the revised MS ([Pages 10-12, lines 205-254](#)). Thanks for your understanding!

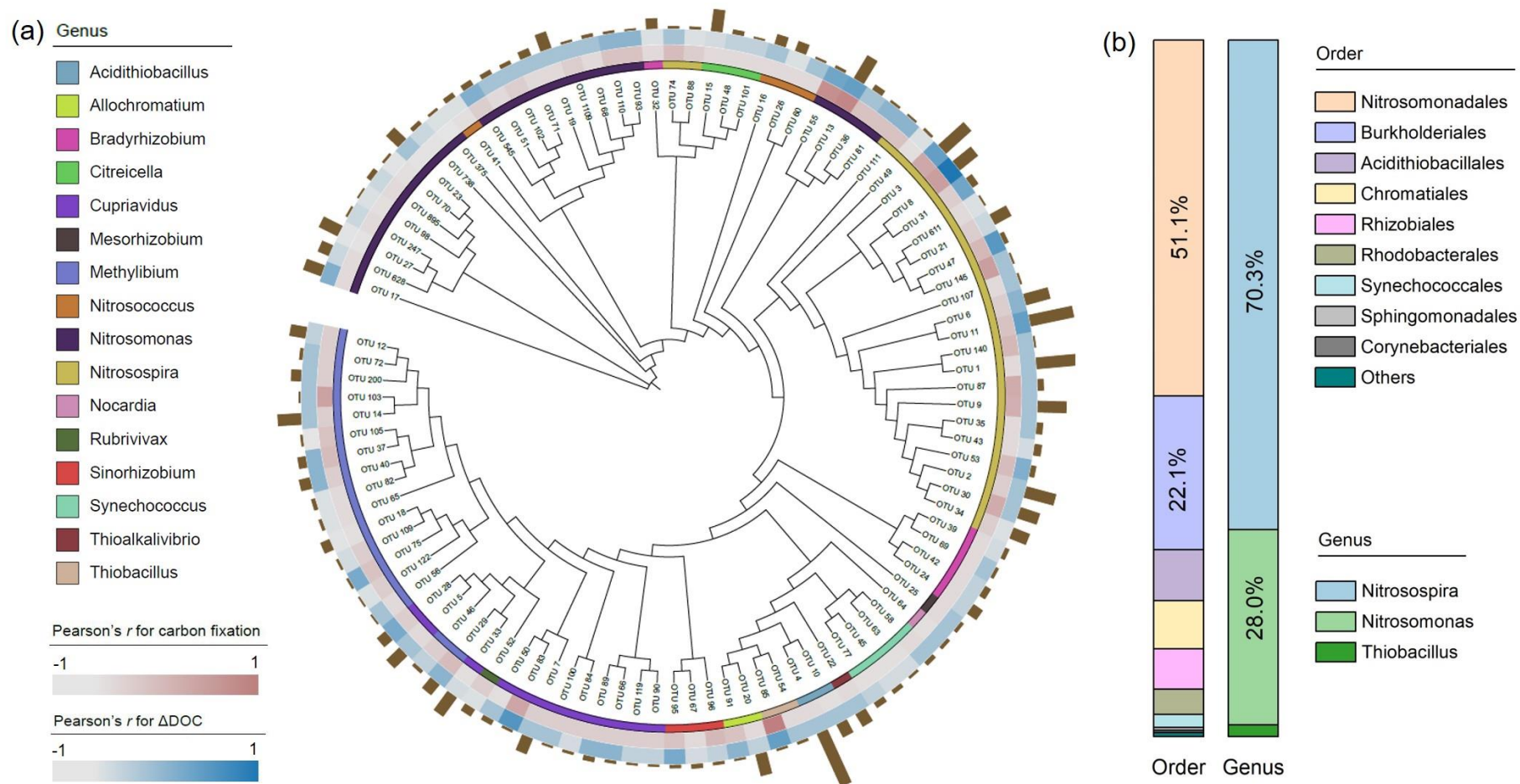


Fig. R9. Phylogeny, relative abundances of chemoautotrophs encoding the *cbbL* gene and their associations with microbial C fixation. a, The phylogenetic relationships of chemoautotrophic bacteria containing the *cbbL* gene. Only OTUs with relative abundances > 0.1% were included

in the tree. The color in the first ring represents the different genera of chemoautotrophs. The second and third rings show the relationships of C fixation rates and ΔDOC (% increase) with the corresponding abundances of OTUs, where the variations of color indicate the strength of the correlations. The outside bar of the third ring corresponds to the relative abundances of OTUs. **b**, The first pillar shows the relative abundances of chemoautotrophs at the order level, and the second pillar shows the relative abundances of *Nitrospira*, *Nitrosomonas*, and *Thiobacillus* genera within the *Nitrosomonadales* order. Dark C fixation was determined based on the $\text{NaH}^{14}\text{CO}_3$ assimilation experiment (Berg et al., 2015) for water samples collected in summer of 2020. ΔDOC (% increase) was examined by using a 28-day incubation determined based on DOC concentration measurement (Vonk et al., 2015) for water samples collected in summer of 2020.

[Comment 8] *The rates of nitrification should be measured, as nitrifiers are claimed to take advantages. Also, nitrification rates should have significant correlation with dark C fixation to confirm nitrifiers really dominant chemoautotrophs.*

[Response] Following the reviewer's comments, we measured the rates of nitrification in the whole profile waters of thermokarst lakes based on ^{15}N -isotope incubation experiments (Overholt et al., 2022). Our results showed that the nitrification rates ranged from 0.26-1.84 $\mu\text{mol L}^{-1} \text{d}^{-1}$ (Fig. R10). Moreover, nitrification rates were positively correlated with dark C fixation rates and the increased proportion of DOC during the incubation period (Fig. R10), confirming that nitrifiers are really dominant chemoautotrophs in the thermokarst lakes. We have added the data involving the rates of nitrification and re-drawn Figure 6 in the revised MS to prove that the dark C fixation was primarily fueled by nitrification (Page 53, lines 987-999).

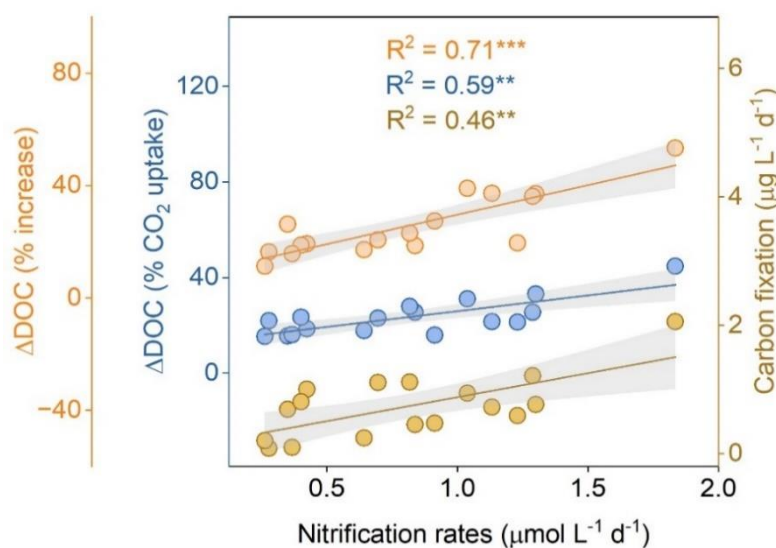


Fig. R10. Nitrification rates in the whole water column of thermokarst lakes and their associations with dark C fixation. Nitrification rates of water samples in each layer collected from thermokarst lakes were determined based on ^{15}N -isotope incubation (Overholt et al., 2022). Notably, considering the high experimental cost, we selected a total of 36 water samples from the investigated thermokarst lakes collected in summer of 2024 and then determined the nitrification rates for each sample, which was same to the samples used for ^{14}C -labelling incubation. ΔDOC (% increase) and ΔDOC (% CO_2 uptake) denote the increased percentage of DOC during incubation

determined based on DOC (Vonk et al., 2015) and CO₂ measurements (Wickland et al., 2012), respectively. Dark C fixation rates were determined on the basis of ¹⁴C-labelling incubation (Berg et al., 2015). The solid lines denote the fitted ordinary least-squares model, and the gray areas indicate the 95% confidence intervals. ** $P < 0.01$ and *** $P < 0.001$.

[Comment 9] The gene abundances are not always equal to the real activities of functional microbes. Thus, DNA-SIP or metatranscriptome should be conducted to further reveal the microbial mechanisms.

[Response] We agree with the reviewer's point that "*the gene abundances are not always equal to the real activities of functional microbes*" and also acknowledge the reviewers' opinion that DNA-SIP or metatranscriptome are better solutions to reveal the microbial dark C fixation mechanisms. Frankly speaking, given the actual conditions in the field (*i.e.*, long sampling distance and crude experimental conditions), it is difficult for us to conduct microbial analyses using the above two methods. Specifically, this study conducted large-scale sampling in the field, each round of sampling campaign takes lots of time due to the long distance and rough road. For example, in 2024, we required 3 to 4 weeks to collect water samples from 40 thermokarst lakes along a ~1,100-km permafrost transect. Due to this situation, metatranscriptomic sequencing could not be performed in this study given that the extracted RNA for metatranscriptomic analysis cannot be preserved for a long time. In addition, both metatranscriptome and DNA-SIP require large amounts of RNA and DNA (approximately 100 L per water sample is required) (Overholt et al., 2022), it is difficult for us to collect enough RNA and DNA in the field due to crude experimental conditions and inclement weather.

We have mentioned this shortcoming in the Discussion section of the revised MS as follows: "*Although this research advances our understanding about patterns and drivers of DOC dynamics in thermokarst lakes across the Tibetan Plateau, there remain*

some limitations which need to be tackled in future studies. First, owing to the logistical constraints of large-scale sampling and the challenges of preserving samples under remote and harsh field conditions, this study was limited to DNA-based analyses, including high-throughput sequencing and metagenomics, to characterize chemoautotrophs in the studied thermokarst lakes. While DNA-level data shed insights into the potential functional capacity of microbial communities, they do not directly capture in situ activity because that the gene abundances are not always equal to the real activities of functional microbes” (Page 12, lines 256-265). Thanks for your understanding!

Thanks again for this reviewer’s insightful and professional review. As mentioned above, we re-collected water samples and sediments from thermokarst lakes in 2024, and **illustrated that chemoautotrophic C fixation occurred in the whole water column and the corresponding sediments, whether in the DOC decreasing lakes or DOC increasing lakes.** Moreover, we performed metagenomic sequencing and demonstrated that **chemoautotrophs in thermokarst lakes mainly fixed C through CBB cycle rather than 3-HP/4-HB cycle.** Furthermore, we **calculated the contribution of dark C fixation to DOC accumulation** and discussed why the contribution was low. In addition, we **determined the rates of nitrification based on ¹⁵N-isotope incubation and illustrated that they were positively correlated with dark carbon fixation,** confirming that nitrifiers were dominated in chemoautotrophs. During these revisions, **we added another nine supplementary figures and four supplementary tables to support the main conclusion drawn in this study.** Overall, **we tried our best to resolve the reviewer’s concerns by devoting more than one year to conduct field sampling and indoor experiments** (including ¹⁴C-labelled incubation for water samples and sediments, bioincubation experiments based on DOC and CO₂ measurements, metagenomic sequencing, and ¹⁵N-isotope incubation experiments). By doing so, we feel that our revised MS has been greatly improved and expect that the reviewer will be satisfied with the revised MS. Thank you!

Responses to Reviewer #2

*[Comment 1] In this study, the authors investigated the potential for chemoautotrophic C fixation in thermokarst lakes. They combined biodegradable DOC experiments and ¹⁴C-labeling bicarbonate assimilation incubations using water samples from 188 thermokarst lakes along a 1,100-km permafrost transect across the Tibetan Plateau. They found that 1/3 of the studied thermokarst lakes experienced significant C fixation dominated by chemoautotrophs resulting in DOC accumulation rather than loss during the incubation period. They also found that C fixation is driven primarily by nitrifying microbes using the *cbbL* gene and corresponding Calvin-Benson-Bassham cycle. They propose that chemoautotrophic C fixation predominates in some thermokarst lakes, partially offsetting substantial C emissions caused by permafrost thaw and weakening of the permafrost C-climate feedback. Overall, this is an interesting manuscript focused on chemoautotrophic C fixation in thermokarst lakes, which has been largely ignored in the literature. However, while the authors' use of incubation experiments and quantitative PCR is commendable, I believe that their experimental design and broad interpretations requires additional validation before I can recommend this paper for publication.*

[Response] Thanks for the reviewer's insightful comments. During the past one year, we tried our best to address the issues pointed out by the reviewer and made the following major changes:

- We re-collected water samples in the whole water column of thermokarst lakes and throughout spring, summer and autumn (2024), which were then used to explore the dissolved organic carbon (DOC) dynamics so as to address the limitation of method based on a single sampling time point in our initial research.
- We conducted another bioincubation experiment of water samples via gas measurements (CO₂), providing direct experimental validation to demonstrate that chemoautotrophic fixation could offset carbon (C) emissions.
- We added ¹⁴C-labelled incubation to explore whether the dark C fixation occurred in thermokarst lakes with DOC loss.

- We performed metagenomic sequencing to demonstrate that the dark C fixation was processed mainly through the CBB cycle rather than other chemoautotrophic C fixation pathways such as 3-HP/4-HB, WL, rTCA, 3-HP and DC/4-HB.

By addressing these comments, we feel that the revised MS has been greatly improved. Details modifications please our responses to the following comments.

[Comment 2] Noteworthy Results: This study was observational by design, yet its experimental results highlight the potential for chemoautotrophic C fixation in a subset of thermokarst lakes across the Tibetan Plateau. There are two particularly noteworthy results, (1) identifying that thermokarst lakes in swamp meadow have higher C fixation rates than lakes in alpine steppe or alpine meadows, and (2) DOC fixation was driven primarily by nitrification with nitrifying bacteria being the dominant group of chemoautotrophs in these lakes.

[Response] Many thanks for the reviewer's positive comments!

[Comment 3] Validity: The authors' primary conclusion that chemoautotrophic C fixation is the predominant microbial process that occurs in a subset of thermokarst lakes can only be partially validated by their experimental design and subsequent results. It is important to note that their methodological approach limits their primary conclusion as well as their broader conclusions regarding the partial offsetting of substantial C emissions caused by permafrost thaw. Specifically, their incubation results are presented in a binary fashion such that an individual thermokarst lake either degrades DOC or fixes DOC. They conclude that 1/3 of sampled lakes fix DOC based on a single sampling time point using 100 mL of filtered surface lake water in a 28-day dark incubation experiment. The reality is much more nuanced, where there is likely DOC degradation and fixation occurring simultaneously within each lake that varies seasonally and by depth based on the changing composition of the microbial community and abiotic factors. Their experimental design cannot capture these nuances and their conclusions are too simplified to be applied broadly across the sampling region. At best,

their results can only suggest that chemoautotrophic C fixation is one of potentially many possible microbial processes affecting DOC concentrations in thermokarst lakes.

[Response] Very good comment! We agree with the reviewer's point that "*the reality is much more nuanced in thermokarst lakes, where there is likely DOC degradation and fixation occurring simultaneously within each lake that varies seasonally and by depth based on the changing composition of the microbial community and abiotic factors*".

To address this issue, we selected out 40 of the 188 thermokarst lakes collected in 2020, and **re-collected water samples in the whole water column from these selected 40 lakes in spring (sampling from 25th-May to 15th-June), summer (sampling from 20th-July to 26th-August), and autumn (sampling from 5th-October to 20th-October) in 2024 (Fig. R1).** Specifically, water samples were collected from the **0-20, 20-50, 50-80, and 80-110 cm water layers** according to different lake depths (Table R1). In spring, 70 samples were collected from these 40 lakes, 64 samples were collected in summer, and 72 samples were collected in autumn, with a total of 206 water samples being collected.

Based on these newly collected samples, **we conducted the bioincubation experiment** to determine the variations of DOC concentration (Vonk et al., 2015). Our results revealed that 20.0% of lakes accumulated organic C in spring, 42.2% of lakes increased organic C in summer, 37.5% of lakes enhanced organic C in autumn. In total, 33.0% of lakes increased the concentration of DOC throughout these three seasons, which was similar to the proportion (36.7%) of observed lakes occurring with DOC accumulation in 2020 (original sampling described in previous version of this manuscript). Moreover, we also observed the phenomenon of DOC accumulation occurred in the surface and subsurface water of partial thermokarst lakes (Fig. R2a-b). To confirm the reliability of bioincubation experiment mentioned above, **we conducted another biodegradable DOC experiment based on CO₂ measurement** (Wickland et al., 2012), and found similar results to those determined based on the variations of DOC concentration (Fig. R2c-d). **These two lines of evidence proved the existence of dark C fixation process**

in the whole water column and throughout all the three seasons, demonstrating that partial thermokarst lakes experienced the process that dark C fixation exceeded DOC degradation, resulting in DOC accumulation rather than loss. Based on these results, we agree with the reviewer's point that "*chemoautotrophic C fixation is one of potentially many possible microbial processes affecting DOC concentrations in thermokarst lakes*" due to the fact that "*DOC degradation and dark C fixation could occur simultaneously within each lake*". In the DOC decreasing lakes, they experienced the process that DOC degradation exceeded dark C fixation, while in the DOC increasing lakes, they suffered the process that dark C fixation exceeded DOC degradation. We have clearly stated this point in the revised MS, and provided the related data and re-drawn the figures involving the biodegradable DOC experiments (based on two methods) (Pages 5-6, lines 101-119; Pages 43-44, lines 913-925; Page 4, lines 15-27 in the supplementary information of revised MS).

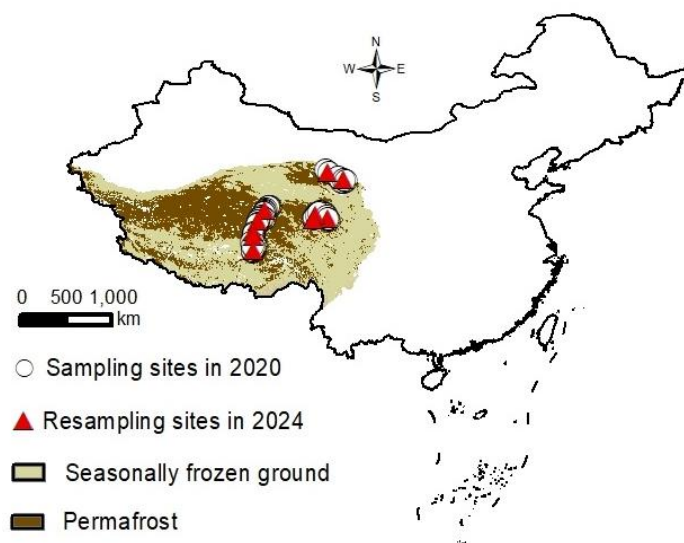


Fig. R1. Distribution of sampling sites across the Tibetan Plateau. The white circles denote the sampling sites of thermokarst lakes collected in 2020. The red triangles indicate the resampling sites collected in 2024. In 2020, we collected surface water samples (0-20 cm) from the 188 thermokarst lakes within 46 sites. In 2024, we collected water samples in the whole water column from the 40 thermokarst lakes within 10 sites. We also collected surface sediments (0-1 cm) from these selected 40 lakes in 2024. The map of permafrost distribution on the Tibetan Plateau is derived from published literature (Zou et al., 2017), and created by the authors using ArcMap 10.2 (Environmental Systems Research Institute, Inc., Redlands, USA).

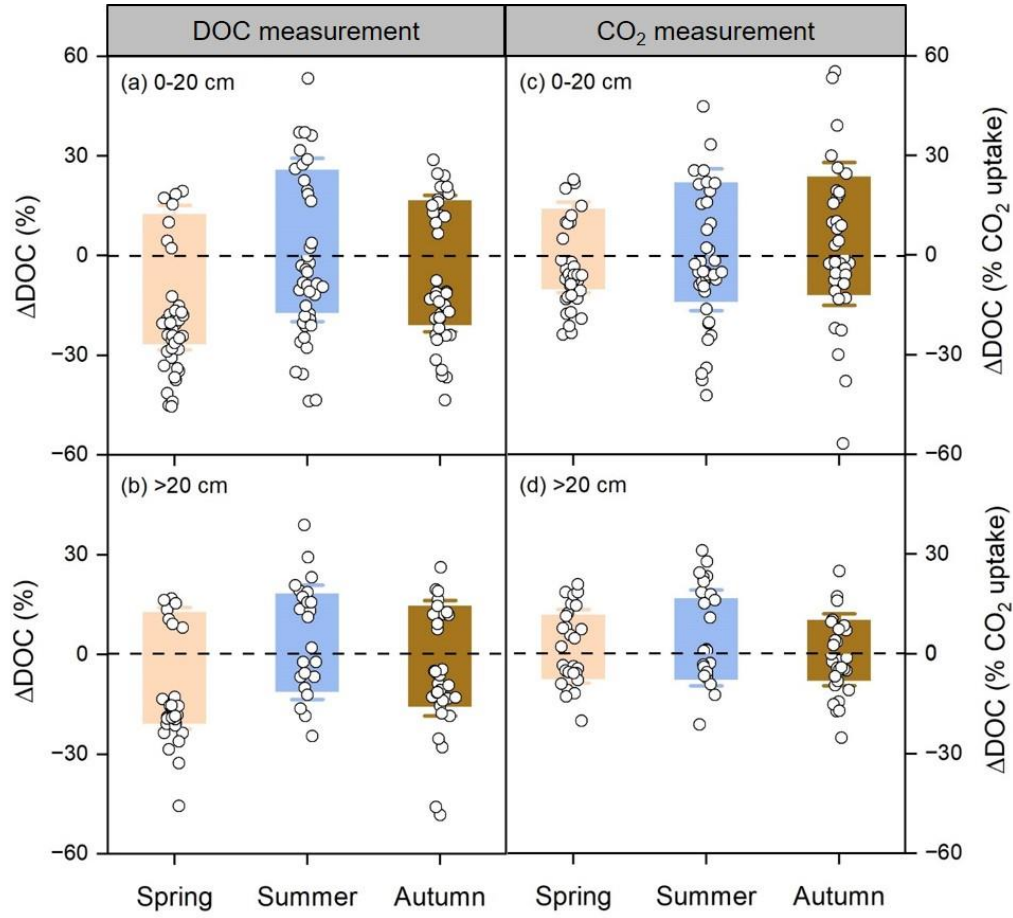


Fig. R2. Changes in DOC of thermokarst lakes during the incubation across different seasons of 2024. **a, b,** The changed percentage of DOC in the surface (a) and subsurface water (b) of thermokarst lakes throughout spring, summer and autumn during a 28-day incubation determined based on the DOC concentration variations (Vonk et al., 2015). **c, d,** The changed proportion of DOC in the upper (c) and lower water (d) of thermokarst lakes throughout the three seasons determined based on the CO₂ absorption or emissions (Wickland et al., 2012). If ΔDOC was positive, it represented the increased proportion of DOC during the incubation. By contrast, if ΔDOC was negative, it indicated the proportion of DOC loss during the incubation. The circles distributed on the bar indicate the data of examined water samples in each thermokarst lakes. The dashed lines in panels (a and b) distinguish water samples in thermokarst lakes occurring with DOC accumulation and loss. Notably, considering ~85% of thermokarst lakes with depth below 50 cm (Table R1), we mainly divided the investigated lakes into surface layer (0-20 cm) and sublayer (> 20 cm).

Table R1. Characteristics of the 40 investigated thermokarst lakes collected from 10 sites across the Tibetan Plateau in 2024.

Site code	Lake code	Latitude (°N)	Longitude (°E)	Altitude (m)	Lake depth (cm)		
					Spring	Summer	Autumn
Site 1	1-1	38.68	99.28	3439	19.2	18.1	11.5
	1-2				20.1	20.3	18.3
	1-3				18.5	19.4	13.4
	1-4				15.3	18.5	12.1
Site 2	2-1	37.99	100.83	3230	15.5	29.3	40.6
	2-2				14.8	33.5	35.2
	2-3				22.5	26.4	30.7
	2-4				13.8	14.4	28.3
Site 3	3-1	34.26	99.20	4353	14.3	56.2	43.5
	3-2				15.5	57.1	46.4
	3-3				11.9	54.3	49.1
	3-4				18.6	49.4	48.5
Site 4	4-1	34.64	98.03	4181	13.3	18.6	19.5
	4-2				29.2	16.7	17.3
	4-3				20.6	17.5	18.1
	4-4				17.1	19.4	19.8
Site 5	5-1	34.18	97.78	4557	82.5	44.3	40.5
	5-2				76.1	49.5	35.3
	5-3				26.7	36.1	33.1
	5-4				34.8	47.8	45.8
Site 6	6-1	35.11	93.04	4664	42.8	43.5	46.8
	6-2				46.5	48.3	49.9
	6-3				49.6	35.2	40.1
	6-4				20.5	19.9	36.5
Site 7	7-1	34.10	92.34	4694	42.6	19.6	38.1
	7-2				28.9	18.7	30.6
	7-3				50.5	17.3	42.7
	7-4				31.5	20.2	30.1

Site code	Lake code	Latitude (°N)	Longitude (°E)	Altitude (m)	Lake depth (cm)		
					Spring	Summer	Autumn
Site 8	8-1	33.10	91.90	4887	86.1	68.8	73.6
	8-2				106.3	81.8	76.2
	8-3				76.8	14.6	32.1
	8-4				110.6	79.3	78.1
Site 9	9-1	32.63	91.85	5003	47.1	33.7	38.2
	9-2				19.8	35.9	32.2
	9-3				28.9	18.8	22.3
	9-4				27.8	19.2	19.5
Site 10	10-1	31.31	91.86	4486	46.8	38.7	40.1
	10-2				36.5	32.6	32.5
	10-3				32.8	17.9	13.7
	10-4				19.4	15.3	12.3

[Comment 4] Their second conclusion that chemoautotrophic C fixation occurs predominately through nitrification using the cbbL gene and Calvin-Benson-Bassham cycle can also be only partially validated by their methodological approach. The use of specific primers for genes encoding chemoautotrophic fixation pathways (3-HP/4-HB and CBB cycles) rather than a metagenomic approach that captures the full potential of genes available to the community limits the full scope of their analysis. The authors have done a fine job linking the abundance of cbbL genes with nitrifying bacteria in their incubations by drawing on previous literature, but without metagenomic insights, they may be missing other important chemoautotrophic components in the community that their PCR-based approach cannot capture.

[Response] Very good comment! Following the reviewer's comment, **we performed metagenomic sequencing using the water samples of thermokarst lakes collected in 2024**, and did not detect the functional genes involved in the 3-HP/4-HB cycle in 92% of examined samples. Moreover, the abundance of functional genes involved in the 3-HP/4-HB cycle was less than 0.1, which was much lower than that involved in the CBB cycle (Fig. R3a). Furthermore, the abundances of functional genes involved in

chemoautotrophic C fixation pathways, such as WL, rTCA, 3-HP, 3-HP/4-HB, and DC/4-HB, were not only low, but also not related to the dark C fixation rates (Fig. R4). By comparison, the genes in CBB cycle were positively associated with the dark C fixation rates and increased proportion of DOC during the incubation (Fig. R3b).

Additionally, by conducting metagenome-assembled genomes (MAGs), we obtained a total of 17 MAGs involved in the CBB cycle, of which 16 encoded form I Rubisco (cbbL), and only one encoded form II Rubisco (cbbM) (Fig. R5). Based on real-time fluorescence quantitative PCR, we found that the cbbL gene abundance was more than ten times higher than that of the cbbM gene (Fig. R6). These two lines of evidence combined with the significant correlation between the cbbL gene copies and dark C fixation provided stronger evidence that the CBB cycle was mainly carried out through the form I enzyme protein encoded by the cbbL gene. Overall, our results derived from metagenomic sequencing also illustrated that the dark C fixation was performed mainly through the CBB cycle rather than other chemoautotrophic C fixation processes. We have added the related results and discussion involving the metagenomics to the revised MS (Pages 7-9, lines 153-190).

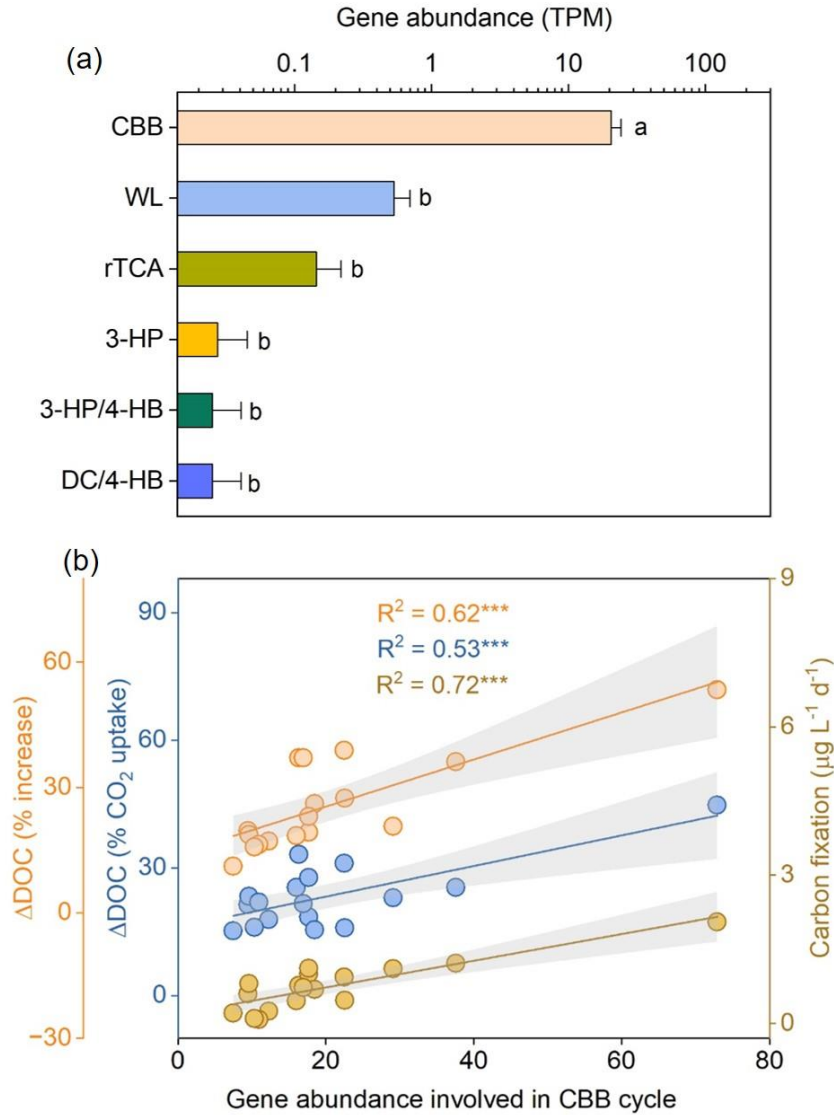


Fig. R3. Normalized abundances of functional genes involved in chemoautotrophic C fixation pathways and their associations with DOC accumulation and dark C fixation in thermokarst lakes during the incubation. **a**, Normalized abundances of genes involved in C fixation pathways of CBB, WL, rTCA, 3-HP, 3-HP/4-HB and DC/4-HB. **b**, Relationships of gene abundance in CBB cycle with ΔDOC (% increase), ΔDOC (% CO₂ uptake) and dark C fixation rates in thermokarst lakes. ΔDOC (% increase) and ΔDOC (% CO₂ uptake) denote the increased percentage of DOC during the incubation determined based on DOC (Vonk et al., 2015) and CO₂ measurements (Wickland et al., 2012) for water samples collected in summer of 2024, respectively. Dark C fixation rates of thermokarst lakes were determined on the basis of ¹⁴C-labelling incubation for water samples collected in summer of 2024 (Berg et al., 2015). CBB,

Calvin-Benson-Bassham cycle; WL, Wood-Ljungdahl pathway; rTCA, reverse glycine cleavage pathway; 3-HP, 3-hydroxypropionate bicycle; 3-HP/4-HB, 3-hydroxypropionate/4-hydroxybutyrate cycle; DC/4-HB, 4-hydroxybutyrate/dicarboxylic acid cycle. Different lowercase letters denote significant differences ($P < 0.05$) among the respective groups by one-way ANOVAs with LSD multiple comparisons. The solid lines denote the fitted ordinary least-squares model, and the gray areas indicate the 95% confidence intervals. Notably, statistically significant relationships of functional genes in CBB cycle with ΔDOC (% increase), ΔDOC (% CO_2 uptake) and dark C fixation in thermokarst lakes during the incubation remained stable even removing the rightmost point in panel (b). *** $P < 0.001$.

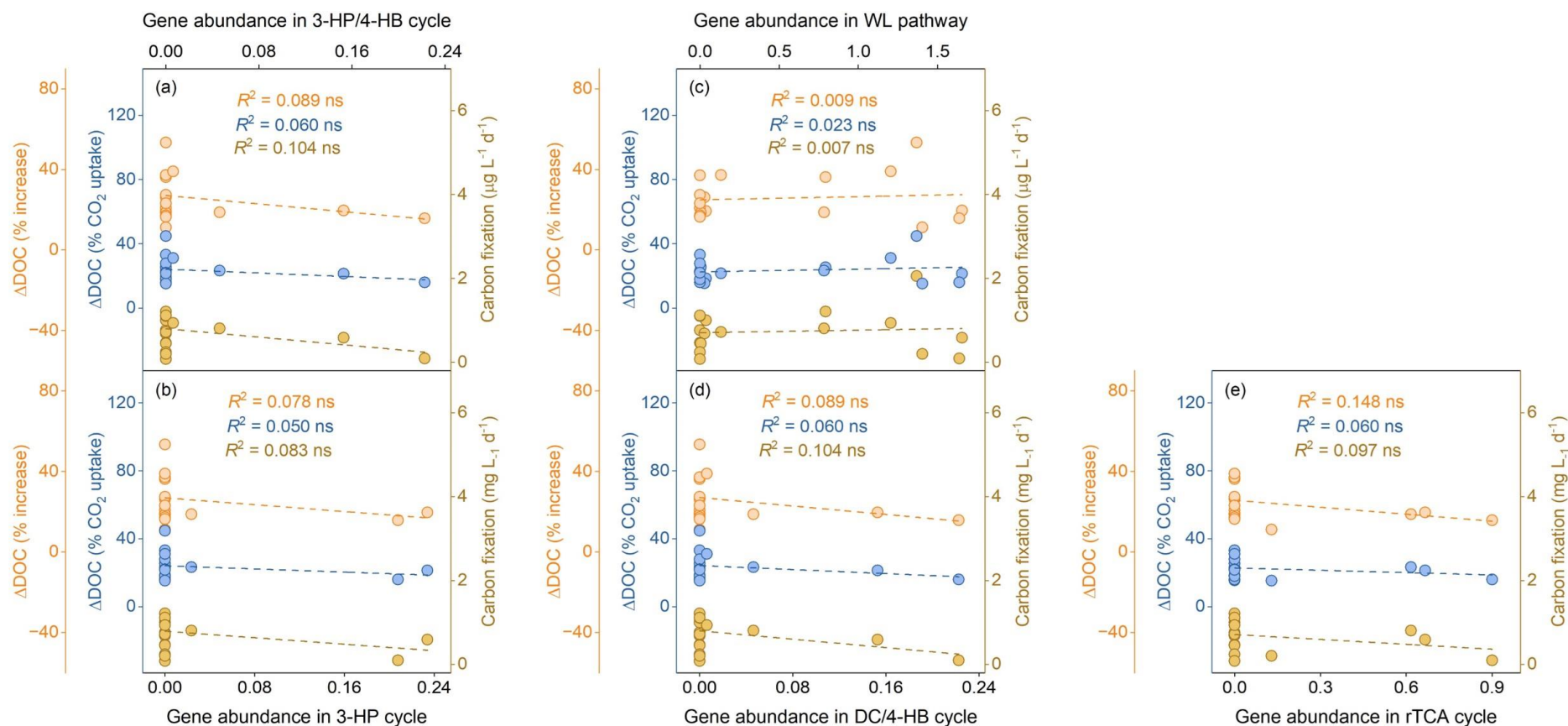


Fig. R6. Relationships of abundances of functional genes involved in chemoautotrophic C fixation pathways with the dark C fixation in thermokarst lakes. a-e, Relationships of genes involved in 3-HP/4-HB (a), 3-HP (b), WL (c), DC/4-HB (d), and rTCA (e) with ΔDOC (% increase), and ΔDOC (% CO_2 uptake) and dark C fixation rates in thermokarst lakes. ΔDOC (% increase) and ΔDOC (% CO_2 uptake) denote the increased

percentage of DOC during the incubation determined based on DOC (Vonk et al., 2015) and CO₂ measurements (Wickland et al., 2012) for water samples collected in summer of 2024, respectively. Dark C fixation rates of thermokarst lakes were determined on the basis of ¹⁴C-labelling incubation for water samples collected in summer of 2024 (Berg et al., 2015). 3-HP/4-HB, 3-hydroxypropionate/4-hydroxybutyrate cycle; 3-HP, 3-hydroxypropionate bicycle; WL, Wood-Ljungdahl pathway; DC/4-HB, 4-hydroxybutyrate/dicarboxylic acid cycle; rTCA, reverse glycine cleavage pathway. ns, $P > 0.05$.

Fig. R5. Phylogeny and number of putative chemoautotrophic MAGs. **a**, Approximately maximum-likelihood phylogenetic tree was constructed based on single-copy concatenated protein using GTDB-Tk (Chaumeil et al., 2019). Colored pentagrams marked on the branch denote the predicted C fixation pathways, including CBB cycle (red), rTCA cycle (blue), and WL pathway (yellow). The first and second rings show the completeness and contamination of MAGs in this study. The outside bar of the second ring corresponds to the mean relative abundance of MAGs in thermokarst lakes collected in summer of 2024. **b**, Colored squares denote that MAGs for chemoautotrophic C fixation were detected in water samples. MAGs, Metagenome-assembled genomes; CBB, Calvin-Benson-Bassham cycle; rTCA, reverse glycine cleavage pathway; WL, Wood-Ljungdahl pathway.

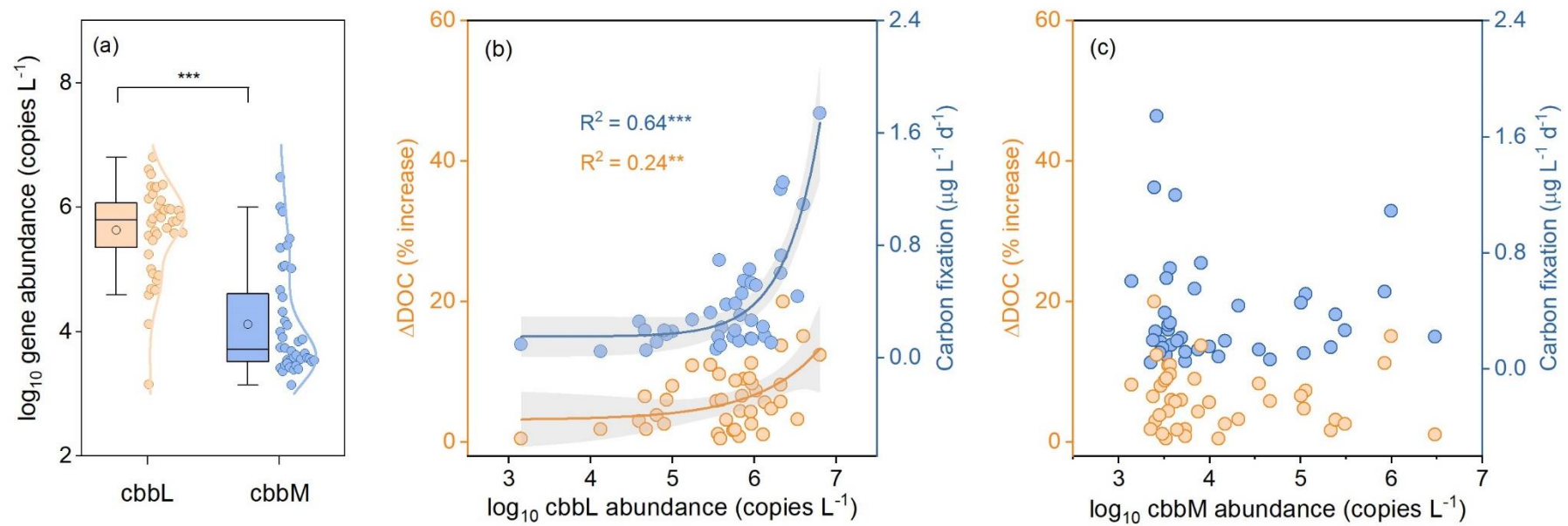


Fig. R6. Abundances of the *cbbL* and *cbbM* genes and their associations with dark C fixation. **a**, Differences in abundances between the *cbbL* and *cbbM* genes in thermokarst lakes where DOC accumulated. **b**, **c**, Relationships of the *cbbL* (**b**) and *cbbM* (**c**) genes with C fixation rates (orange line) and ΔDOC (% increase; blue line), respectively. Exponential fittings are used in panel (**b**) with the grey areas corresponding to 95% confidence intervals. ΔDOC (% increase) was examined by using a 28-day incubation determined based on DOC concentration measurement (Vonk et al., 2015) for water samples collected in summer of 2020. Dark C fixation was determined based on the $\text{NaH}^{14}\text{CO}_3$ assimilation experiment (Berg et al., 2015) for water samples collected in summer of 2020. Significant differences between the *cbbL* and *cbbM* gene abundances were determined based on a paired *t*-test. $^{**}P < 0.01$ and $^{***}P < 0.001$.

[Comment 5] Significance: The significance of this research is the discovery of substantial potential for chemoautotrophic C fixation by nitrifying bacteria in thermokarst lakes. As the authors mention, chemoautotrophic fixation of DOC has been historically understudied relative to DOC degradation processes following permafrost thaw. This manuscript is a great first step providing proof of concept for potential chemoautotrophic fixation activity and the authors did well to determine a potential genetic mechanism for chemoautotrophy through the increased abundance of nitrifying functional genes (cbbL) and associated bacterial taxa. However, the lack of repeated measures within each lake or across seasons (and years) limits the validation of this response to a one-off observation within each individual lake across the sampling gradient. The methodological lack of validation and the binary nature of their analysis limits the interpretation of these results and the authors' conclusion that chemoautotrophic C fixation could offset substantial C emissions caused by permafrost thaw. These limitations on the interpretation and scalability of the results weakens the broader significance of this research and whether it merits publication in Nature Communications.

[Response] Very good comment! As mentioned above (**response to [Comment 3]**), to address the reviewer's concern, we selected out 40 of the 188 thermokarst lakes collected in 2020, and **re-collected water samples from these selected 40 lakes in spring, summer, and autumn of 2024** (Fig. R1), with a total of 206 water samples being collected. By using these newly collected samples, we conducted bioincubation experiment based on determining the variations of DOC concentration (Vonk et al., 2015). Our results illustrated that 20.0% of lakes accumulated organic C **in spring**, 42.2% of lakes increased organic C **in summer**, 37.5% of lakes enhanced organic C **in autumn**. In total, 33.0% of lakes increased the concentration of DOC throughout these three seasons, which was similar to the proportion (36.7%) of observed lakes occurring with **DOC accumulation in 2020** (original sampling described in previous version of this manuscript).

To address the reviewer's concern about the methodological lack of validation and the binary nature of analysis, [we performed another biodegradable DOC experiment using the modified method based on CO₂ measurement](#) (Wickland et al., 2012). Our results revealed that a total of 39.3% of lakes increased organic C via fixing CO₂ throughout all the three seasons. This observation was similar to those determined based on the variations of DOC concentration during incubation (Fig. R2c-d). These two methods confirmed that partial thermokarst lakes experienced the process that chemoautotrophic C fixation exceeded DOC degradation, which could offset substantial C emissions caused by permafrost thaw.

Overall, through re-collecting water samples across seasons and conducted another bioincubation experiment based on CO₂ measurement, this study overcame the limitations on the interpretation and scalability of the results in the original MS, which strengthened the broader significance of this research and merited publication in a top journal like *Nature Communications*. We have clearly stated these points in the revised MS (Pages 5-6, lines 101-119).

[Comment 6] Data and Methodology: I have several concerns regarding the methodology. My first concern is that the authors limited their radiolabeled bicarbonate incubations and all microbial analyses to the 40 randomly selected lakes that accumulated DOC in their biodegradable DOC experiment. I understand that costs were an issue, but it would have been nice to see results from at least one and preferably several lakes where DOC concentrations declined, to serve as a control. What is the microbial composition of those 119 lakes where DOC degradation increased? Are there chemoautotrophic taxa in those lakes? Can we use microbial 16S sequencing as a screening mechanism for determining if a thermokarst lake has the potential to fix DOC through chemoautotrophic pathways? The lack of data from 2/3 of the lakes makes it difficult to validate the significance of the results presented in the manuscript.

[Response] Very good point! Following the reviewer's comments, we performed the following two analyses. First, we conducted ^{14}C -labelled incubation for the thermokarst lakes suffering DOC loss. Our results showed that the dark C fixation process also existed in DOC decreasing lakes, with rates ranging from 0.04 to 0.76 $\mu\text{g C L}^{-1} \text{ d}^{-1}$ with a mean of $0.44 \pm 0.05 \mu\text{g C L}^{-1} \text{ d}^{-1}$ (Fig. R7a). In the remaining thermokarst lakes with the occurrence of DOC accumulation, the C fixation rates varied from 0.10 to 2.05 $\mu\text{g C L}^{-1} \text{ d}^{-1}$, with a mean of $0.74 \pm 0.12 \mu\text{g C L}^{-1} \text{ d}^{-1}$ (Fig. R7b). Interestingly, the C fixation rates of thermokarst lakes occurring with DOC accumulation was significantly higher than those occurring with DOC degradation (Fig. R7c). In the lakes occurring with DOC loss, we found no significant correlation between the dark C fixation rates and the decreased percentage of DOC during the incubation period (Fig. R7a). By contrast, in the lakes exhibiting DOC accumulation, the increased percentage of DOC was positively correlated with the rates of dark C fixation (Fig. R7b). These results indicated that the dark C fixation had limited effects on the DOC dynamics of lakes occurring with DOC loss, but could exert crucial roles in mediating the DOC increasing lakes.

Second, we conducted 16S rRNA amplicon sequencing for the water samples collected from 188 thermokarst lakes in 2020 by using 515F/806R primers (Kang et al., 2023). Then we analyzed the characteristics of microbial community composition based on the 16S sequencing data. Our results indicated that microbial community composition of those 119 lakes where DOC degradation mainly consisted of *Betaproteobacteriales*, *Flavobacteriales*, *Micrococcales*, and *Cytophagales* within the order level (Fig. R8a). At the genus level, they were primarily composed of *Flavobacterium*, *Polynucleobacter*, *Candidatus Limnoluna*, and *Sphingorhabdus* (Fig. R8b). Overall, the microbial community composition in DOC decreasing lakes ($n = 119$) was similar to that in DOC increasing lakes ($n = 69$), although significant differences existed in partial of microbial composition between these two groups ($P < 0.05$; Fig. R8a-b). In addition, we found chemoautotrophic taxa (including *Nitrosospira*,

Nitrosomonas, and *Thiobacillus*) in those DOC decreasing lakes, accounting for approximately 4% of the microbial community composition in thermokarst lakes (Fig. R8c). However, there was no significant differences in the relative abundance of chemoautotrophic taxa in thermokarst lakes occurring with DOC loss ($n = 119$) and accumulation ($n = 69$). Such a phenomenon could be ascribed to the fact that it is difficult to precisely quantify the abundance of chemoautotrophs through the universal primers of 16S sequencing (Alfreider et al., 2017). Specific primer (*i.e.*, primer of *cbbL* gene) are required to accurately quantify the aforementioned chemoautotrophs. Thus, microbial 16S sequencing alone cannot be reliably used to screen whether a thermokarst lake harbors microbial communities capable of DOC fixation via chemoautotrophic pathways. Taken together, we demonstrated that dark C fixation process not only occurred in DOC increasing lakes, but also existed in DOC decreasing lakes. 16S sequencing could not be used as a screening mechanism for determining the microbial capacity to fix DOC. These above analyses validated the significance of our results presented in the MS. We have clearly stated this point in the *Results and Discussion* section of the revised MS (Pages 6-7, lines 121-140; Pages 10-11, lines 205-230).

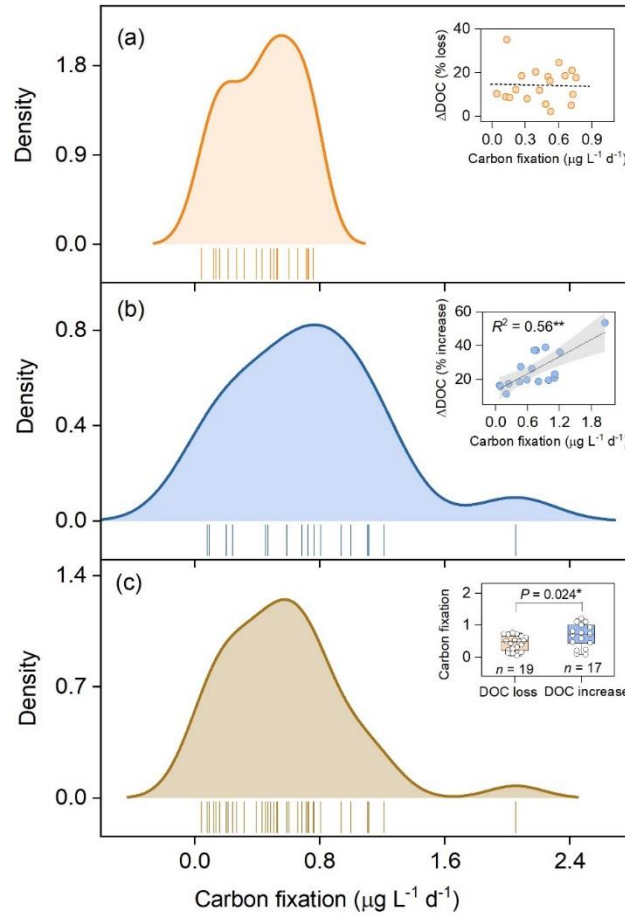


Fig. R7. Dark C fixation rates from the investigated thermokarst lakes collected in summer of 2024. **a, b,** Dark C fixation rates from the thermokarst lakes occurring with DOC degradation (a) and DOC accumulation (b). Inserts in panels (a) and (b) shows the relationships of dark C fixation rates with ΔDOC (% loss) and ΔDOC (% increase) in thermokarst lakes, respectively. The solid lines denote the fitted ordinary least-squares model, and the gray areas indicate the 95% confidence intervals. **c,** Dark C fixation rates in total water samples including those occurring with DOC degradation and accumulation. Inset in panel (c) shows the comparisons of dark C fixation rates among all the thermokarst lakes occurring with DOC loss ($n = 19$) and accumulation ($n = 17$). Notably, considering the high experimental cost, we randomly selected 20 of the investigated thermokarst lakes with a total of 36 water samples being collected, and then calculated the dark C fixation rates for each sample. Dark C fixation rates were determined on the basis of ^{14}C -labelling incubation (Berg et al., 2015). ΔDOC (% increase) and ΔDOC (% loss) denote the increased and decreased percentage of DOC during incubation determined based on DOC measurement (Vonk et al., 2015), respectively. * $P < 0.05$ and ** $P < 0.01$.

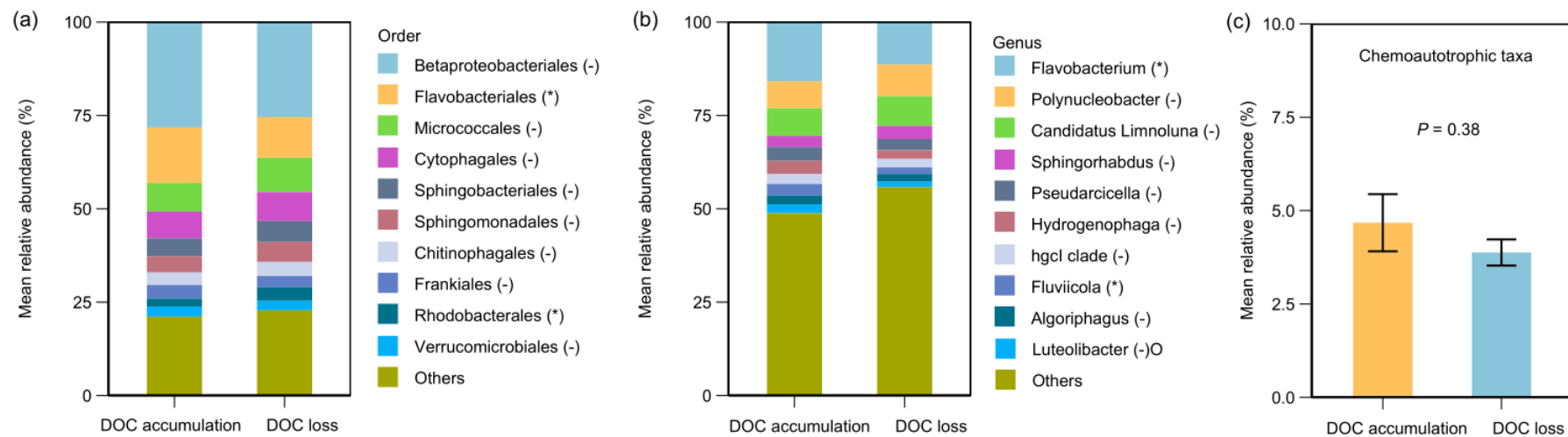


Fig. R8. Microbial community composition in thermokarst lakes determined based on 16S sequencing. **a, b,** The relative abundances of bacterial communities at the order and genus levels, respectively. **c,** Comparisons of the relative abundance of chemoautotrophic taxa in thermokarst lakes occurring with DOC accumulation ($n = 69$) and loss ($n = 119$). Chemoautotrophic taxa refers to the species identified through amplicon sequencing for *cbbL* gene in this study (Fig. 5). The asterisk (*) in the parentheses of the legend indicates significant differences existing in the microbial community composition of lakes occurring with DOC accumulation ($n = 69$) and DOC loss ($n = 119$; $P < 0.05$), while the horizontal line (–) denotes no significant differences among them ($P > 0.05$).

[Comment 7] My second concern is the lack of gas measurements (CO₂) from the incubation experiments. If their broader claim is that chemoautotrophy will fix DOC rather than increase CO₂ emissions through DOC degradation processes, then it seems reasonable that there should have been measurements of CO₂ production from thermokarst lakes where DOC was degraded versus those lakes where DOC was fixed. It is difficult to determine if chemoautotrophic fixation can offset C emissions if there is no experimental validation.

[Response] Following the reviewer's comments, we conducted the gas measurements (CO₂) for water samples in thermokarst lakes. Our results demonstrated that 39.3% of lakes accumulated organic C via fixing CO₂ during incubation (Fig. R2c-d). These results based on gas measurements indicated partial thermokarst lakes occurred with the process that dark C fixation exceeded DOC degradation, which could absorb substantial CO₂ from the atmosphere and provide direct experimental validation that chemoautotrophic fixation can offset C emissions. We have added the method and associated results involving the gas measurements (CO₂) to the revised MS (Page 6, lines 116-119; Pages 18-19, lines 387-409).

[Comment 8] Concluding Thoughts: The authors' efforts to discover and quantify the extent of chemoautotrophic C fixation activity and the microbial mechanisms likely driving this metabolic process in thermokarst lakes is commendable. These results clearly show that this process should be considered as an important microbial factor when assessing the fate of DOC following permafrost thaw. However, this study serves best as an initial proof of concept for chemoautotrophic C fixation by nitrifying taxa. The over-simplification of the results (binary response) and lack of additional sampling time points limits the validation of the primary and broader conclusions as presented. The paper is technically sound regarding the discovery of chemoautotrophic C fixation but remains too speculative to support the broader significance of the results regarding the potential for chemoautotrophic C fixation to partially offset substantial C emissions caused by permafrost thaw and weakening of the permafrost C-climate feedback. The

lack of supporting gas measurements is just one of several limiting factors that make the conclusions of this study too speculative as written.

[Response] Very good comment! As mentioned above, to address the reviewer's concern, we have modified this study in the following two aspects. **First**, we re-collected the water samples throughout the [spring, summer and autumn](#) in 2024 and then analyzed the DOC dynamics in thermokarst lakes (Fig. R1 and Table R1). Our results revealed that chemoautotrophic C fixation predominated in nearly one-third of the studied thermokarst lakes through all the three seasons, resulting in DOC accumulation (Fig. R2). **Second**, we performed the bioincubation experiment of water samples [based on gas measurements \(CO₂\)](#) and found that partial thermokarst lakes occurred with the process that dark C fixation exceeded DOC degradation, which could absorb substantial CO₂ from the atmosphere (Fig. R2c-d). This result [provided direct experimental validation](#) that chemoautotrophic fixation can offset C emissions via fixing CO₂, which also overcame the over-simplification of the results (binary response) based on the method calculating DOC accumulation or loss (Fig. R2a-b). Based on these modifications, this study strengthened the validation of the primary and broader conclusions as presented, and provided stronger evidence to our main arguments.

Overall, we sincerely thank this reviewer's insightful and professional review. These comments inspired us to have a deeper thinking on field sampling, data analyses and results integration, and thus guided us to conduct a thorough revision of the original MS. To address these insightful comments, we re-collected water samples and demonstrated that **chemoautotrophic C fixation of thermokarst lakes occurred in the whole water column and across all the three season**. We also **conducted the bioincubation experiment via CO₂ measurements**, providing direct experimental validation that chemoautotrophic fixation can offset C emissions. We further **performed ¹⁴C-labelled incubation for water samples and found that dark C fixation occurred in thermokarst lakes with DOC loss**. Finally, we **employed metagenomic sequencing to demonstrate that the dark C fixation was processed**

mainly through the CBB cycle rather than other chemoautotrophic C fixation. During these revisions, we added another nine supplementary figures and four supplementary tables to support the main conclusion drawn in this study. Overall, we have tried our best to resolve the reviewer's comments and devoted more than one year to conduct field sampling and indoor experiments (including ^{14}C -labelled incubation for water samples and sediments, bioincubation experiments based on DOC and CO_2 measurements, metagenomic sequencing, and ^{15}N -isotope incubation experiments). By doing so, we feel that our revised MS has been greatly improved and expect that the reviewer will be satisfied with the revised MS. Thank you!

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Responses to Reviewer #2

[Comment 1] I would like to commend the authors for their exceptionally thorough and thoughtful revision. It is clear that a great deal of additional work—both in the field and in the laboratory—was undertaken to address the comments raised in the initial review. The inclusion of multi-season sampling across the water column, CO₂ gas measurements, ¹⁴C-labeled incubations for DOC-decreasing lakes, and metagenomic analyses substantially strengthens the manuscript and provides robust validation of the original conclusions. Specifically: The addition of seasonal and depth-resolved sampling convincingly resolves the concern regarding the single-time-point limitation and provides valuable insights into the temporal and spatial variability of chemoautotrophic C fixation. The incorporation of CO₂ flux measurements offers critical experimental validation for the proposed offsetting of C emissions and demonstrates methodological rigor. The metagenomic analyses are particularly commendable, as they clarify the dominance of the CBB cycle and the role of the *cbbL* gene in dark C fixation, thereby addressing the previous limitation of relying solely on targeted PCR assays. Finally, the inclusion of 16S rRNA and functional gene analyses for both DOC-increasing and DOC-decreasing lakes offers a balanced view of microbial community structure and function across contrasting lake states.

Overall, this revised manuscript represents a substantial improvement over the original version and now provides strong, well-supported evidence for chemoautotrophic carbon fixation in thermokarst lakes. The authors' commitment to addressing the earlier critiques through additional field sampling, gas flux measurements, and metagenomic analyses is commendable and elevates the scientific rigor and impact of the work. I appreciate the authors' thoughtful revisions and am pleased to recommend the manuscript for publication in Nature Communications.

[Response] We sincerely appreciate the reviewer for his recognition and positive evaluation of our revised manuscript. The reviewer's insightful comments have been invaluable in improving the manuscript.