nature portfolio

Peer Review File

Microbial aerotrophy enables continuous primary production in diverse cave ecosystems

Corresponding Author: Professor Chris Greening

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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)
Bay and coworkers strongly increased the quality of the manuscript, and I fully agree with the molecular analysis and observations made in Fig. 1 to 3. Indeed, caves harbor many microorganisms with the genomic potential to use H2, CO2, and CH4 airborne gases. But do they use this potential to produce a substantial amount of chemolithogenic biomass?
I question the claims made about the importance of these substances as energy sources for microorganisms. There might indeed be higher H2 concentrations at the cave's entrance than in the twilight zone, but I do not see any further trend toward the interior of the caves. If so, only the entrance /twilight zones show hydrogen consumption, but the middle and deep zones of the caves do not further contribute to hydrogen oxidation, and the same holds for other gases.
The authors make statements such as high/ substantial rates without bringing their data into context. How much is 1 nM per m^2 and sec or 86.4 µM per sqm and day? Let's get the number into context. The lowest oxygen fluxes on Earth were measured for the South Pacific Gyre- about 0.1 to 1mmol per sqm and day. In the South Pacific Gyre, the oxygen penetration depth is 100 meters. I don't think that anything similar can be observed in the cave. The oxygen consumption in this cave is likely many times higher than the contribution of CO (clearly), hydrogen (for sure in the deeper cave), and methane.
Hence, airborne autotrophy will also result in only traces of biomass production, which will hardly affect the carbon flow in the cave. These are just estimates, but the key experiment to call the hydrogen consumption substantial / and, if at all, is connected to maybe 1% of the oxygen consumption (this would be my estimate) has not been done.
Because of the low rates, cell-specific reaction rates become unrealistically low (Fig 4c-d). Data points such as 10^14 nmol mean the respiration of single molecules per second. This would be difficult to replicate – it would take 7 billion seconds to fix the carbon of the average cell. It is more likely that some few cells are active, but this makes average cell-specific rates odd. It is like having 100000 homeless and Elon Musk at your party and stating, based on average, that all your guests are millionaires.

Because it is impossible to compare the low hydrogen, methane, CO oxidation rates with the missing total oxygen consumption rates, statements such as the last sentence of the abstract are not justified:
"Trace gases sustain a basic level of biodiversity and productivity through a process we define as aerotrophy that is supplemented by organic and inorganic input". In fact, looking at the data, it is rather unlikely that the contribution of these trace gases to the overall microbial activity is very high.
A solution for this abstract could be:
"The energy from airborne trace gasses may support the growth of a yet unquantified share of the cave communities. These organisms may add organic carbon compounds to the energy-limited cave ecosystems. "
The entire paper has an awkward tendentiousness. Whenever a rate shows a bit towards "aerotrophy" it is excessively reported: Just look at Fig. 4ab, discussed in line 250ff. Gas concentrations decreased significantly. I don't see significant value. If you start from twilight, there is simply no decline anymore. Wording like high rates do not match if you cannot compare it with other rates.

Fig. 4. B: Fluxes are never negative according to the graph. But look at the MS. This would also be impossible because it is a logarithmic scale. Numbers of replicates are not given. But data points are up to 5 orders of magnitude different.

Fig. 5: There is much higher methane than CO2 fixation (Fig. 5 c,d) – but the d13C argues against methanotrophy and more for CBB-derived fixed carbon. Of course, most of the organic carbon is not produced by microorganisms but is from sunlight-fixed organic matter.
L244ff: I cannot agree that trace gas consumption is described as high rates.
Fig. 4a: Extended data Figure 7: I do not see a substantial or statistically significant decrease for most curves, except "entrance" and methane in twilight. If so, there is a difference between T0 and T1 – not after. The entrance of the cave might be a difference.
Line 343ff: particularly high hydrogenotrophic carbon fixation rates of 6.7*10^-12 nmol per cell and minute – that does not sound to be a lot

It is unclear what a strong consumption means; see comment above. A change of a fractions of a ppm difference in flux chambers is never a lot. Here and in other cases, I miss comparison with other habitats / or other rates.

Line 364: About half of the carbon should come from CO2 when methane is assimilated. Methane assimilation cannot be three orders of magnitude higher than CO2 assimilation. You may have triggered methane oxidation by the addition of large amounts of 14C-tracer. Unfortunately, methods do not describe 14C-methane oxidation rates. I fear that these high rates derive from incubations with large amounts of 14C: 1 MBq carbon/CH4 is already 0.4 µmol. Please report also on the duration of this incubation.

14CO2-based rate measurement: (line 637) 14C isotope labeling is not the correct terminology here). Note that you measure hydrogen-dependent CO2 fixation rates at H2 concentrations that are 100 times elevated compared to nature. Still, these rates are small.

Extended Fig. 8: Here, a logarithmic curve is not good - or tells a lot – the organisms only start picking on the higher concentrations after a lack phase. This may suggest that environmental trace gas concentrations are hardly used.

Please find a meaningful order of the methods i.e., best use the order of how data appears in the MS

Reviewer #2

(Remarks to the Author)
The authors have addressed nearly all of my concerns and the manuscript is much improved. I still don't like "aerotrophy" as used here, but I seem to be alone in this opinion.

Reviewer #3

(Remarks to the Author)
Comments and Suggestions for Authors

Bay and colleagues have investigated metagenomic capacities and actual biogeochemical cues for active microbial consumption of atmospheric trace gasses, mostly H2, CH4 and CO, in 4 Southern Australia cave systems.
Cave microbiota are compared across longitudinal transects and different compartments of two basalt and two limestone caves in Victoria. With ~1500 high-quality MAGs assembled for many dozens of samples, ~half of the MAGs hosted capacities to consume either one of the different trace gasses. Amongst them, abundant hydrogenotrophic candidate genera within the Actinobacteriota, methanotrophic gammaproteobacterial Ca. Methylocavales, and also some MAGs of enigmatic candidate phyla. Aerotrophic primary production is shown to be substantial throughout most samples, with methane and hydrogen-derived energy delivering enough power-per-cell to allow for continued growth, or at least sustained activities and survival in lack of other substrates. The concept of, at least temporary or partial, aerotrophy as a dominating physiology in caves is a highly interesting addition to our understanding of these underexplored ecosystems. This piece clearly warrants publication in a journal like NatComms. Still, before acceptance, the authors should address the following comments:

1. It Would be good if the results (& discussion) section could start with some basic primers on the nature of the sampled cave systems (basalt and limestone), locations (entrance, longitudinal transects) sample types (biofilms, sediment, …) and overall sample numbers. This is important to grasp the scope of the study, as not every reader will (first or at all?) grind through the M&M or supplement. Just a bit more info on the basic settings of the study, before delving into all the community composition and the metagenomic details.

2. In contrast to the authors notion, caves do typically not contain soil (Fig. 1b). Unless it is washed in or collapses in from above. Please change to "Sediments" in Fig. 1b, as correctly implemented already in other Figs. Also, "soil" must still be replaced on some occasions throughout the text, e.g. L129 or L343.

3. L318: this must be a mistake. Explain that this was just one sample of many dozens with this extremely light signature at MINUS (!) 67.4 permil. And that this is much too depleted to be consistent with the CBB cycle OR any typical methanotrophs growing on light methane. This must point towards a local influx of geogenic, isotopically highly depleted (potentially fossil ?) methane in this one limestone sample. But I agree that especially the biofilm samples in basalt (lighter than -30 permil) are in clear agreement with the CBB

4. There is a clear increase of methanotrophs towards the cave interior. Same as for methane (Esp. for basalt), consumption

rates, fluxes, and per-cell power availability. All of this is not fully consistent with a consumption (and influx) of atmospheric methane via the entrance alone. In addition, there must be a contribution of geogenic methane influx. This should be better discussed, same as its implications for the “aerotrophy” concept as defined by the authors. Geogenic methane is still a trace gas, but does not come from the atmosphere.

5. Along the same line, I am perfectly fine with the authors’ findings on aerotrophic chemosynthesis being ubiquitous and unexpectedly important in cave microbiota. Their overarching conclusions on aerotrophy being able to sustain microbial life clearly beyond mere survival or maintenance energy needs and over longer time spans are convincing and novel.
But still, I suggest to add another grain of caution to some remaining statements on e.g. “dominant primary producers” e.g. line 153. More sampling and data (seasonal or hydraulic event-triggered) would be required to really waterproof such posits, especially for members of the well-known and typically heterotrophic lineage of the Actinobacteriota. At least the option of transient and dynamic heterotrophic, mixotrophic, or aerotrophic growth should not be excluded.
Also the abstract does not offer any conditional or cautioning statements in the latest version. Please add accordingly.

Minor comments:
L97: Consistent taxonomic rigour must be used throughout MS, Text and Figs. E.g. Proteobacteria are correctly named “Pseudomonatdota” etc. Please check https://doi.org/10.1099/ijsem.0.005056 and elsewhere for the latest authoritative nomenclature.

L103: marker genes of basic physiologies?

L116: give abundance range of photosynthesis genes at entrance.

L139: I would refrain from calling them “strategies”, as long as overall activity and rate data are not always available. “Capacities”?

L167: Pseudomonadaceae are NOT within the Actinomycetia, no way! Egibacteracerae, of course, are?

L169: caves appear to…

L 171 here and elsewhere report findings in past tense.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Bay and coworkers significantly improved the paper; however, I still have some questions/ remarks:

L41ff: at rates sufficient to meet community-wide energy needs this need to be tested differently, as it would mean that all biomass would cycle internally, i.e. also biomass of heterotrophs. Below I used your data from CO2 fixation that this is not the case. i.e. in a single example I calculated biomass turnover time of 35,000 years. I simply doubt that this meets the energy demands of all organisms. Maybe: with rates which likely sustain the energy demands of a substantial fraction of the community."

L114: keep consistently present tense when talking about metagenomic data, “encode”, and past when you talk about rates...

L120: be moderate: You can not infer from MAGs that organisms do something: … of cave microorganism that encode enzymes that would allow the use of.. or something

L153: avoid novel here

L169: belong to high-affinity clades of hydrogenases

L183: I discourage the authors from naming uncultured organisms after potential energy sources. i.e., to my knowledge, there are no other hydrogenotrophs within the Pseudonocardinaceae, Egibacteraceae, and Actinomycetia – all of them are rather heterotrophs. Spreading this attribute and manifesting it in their names, based on metagenomic information, seems to be wrong. To my knowledge there are no knallgas bacteria in the Actions. Culture them in the lab, investigate their capabilities, and then this naming is adequate. Instead you may use locations in the name.

L293: Sulfur and iron are only energy substrates when they are reduced. Total S and Fe are not important. What species do you speak of?

L323: Again, I do not see convincing evidence for the growth of these organisms as hydrogen oxidizers. If you want to state this, cultivate the organisms.

L 329ff: The isotopic signatures of most organic matter from cave samples are also consistent with photosynthetic matter, which is no surprise because they use the same C-fixation pathway. Only the basalt biofilm shows robust signs for another carbon source/pathway, which could indeed be methane-derived organic matter.

L357ff: Please contextualize your data – here the reported carbon fixation rates: “Notably, basalt biofilm and sediment microbes mediated higher hydrogenotrophic CO2 fixation activities (5.7 × 10-13 nmol 14C cell-1 min-1) compared to average rates by limestone sediment microbes (5.3 × 10-14 nmol 14C cell-1 min-1). “
Is this a lot? Let’s see how long it would take to build 1.2 % carbon - (12 mg=1 mmolC) this way, 5.3x10^-14 nmol/min per cell * 10^9 cells per g (I guess it is less in this particulate sample – we have 5.3x10^-5 nmol per minute and ccm. How much time does it take to fix 1 mM? To fix 1 mmol of carbon, this takes 1/(5.3*10-5*10-^-6) minutes = 1/(5+10^-11) minutes = 35,870 years!
Therefore, it would take 35,000 years to rebuild the 1% carbon. Although sedimentation rates of organic matter in the caves will also be quite low, it appears that the contribution of CO2 fixation is relatively low.
I ask for similar comments like this in earlier versions.

Since methanotrophs will always fix ~20% of the carbon from CO2, methane carbon fixation can only be four times higher than that. Additionally, we also miss discussing heterotrophic CO2 fixation, which can also be part of the discussion. In total it seems that methane oxidation rates are orders of magnitude higher than H2 oxidation (although so many more organisms encode hydrogen oxidation).

The conclusions are not justified.
L376: “Here we provide extensive metagenomic and biogeochemical evidence that diverse caves are atmospherically powered ecosystems.” See above. Given your data (i.e., H2 consumption, CO2 fixation), I don’t think you can make this claim. As suggested earlier, and implemented before:
Here we provide extensive metagenomic and biogeochemical evidence that diverse caves are atmospherically powered ecosystems.
Here, we provide metagenomic and biogeochemical evidence for the consumption of atmosphere-derived trace gases in cave sediments and biofilms (note that this does not include lakes, walls, etc.). This consumption may feed a yet-to-be-quantified fraction of the microbial community. (or something similar)

“Primary production appears to be driven by highly abundant and active methanotrophs, as well as novel lineages of actinobacterial lithoautotrophs, that continuously use the gases methane, hydrogen, carbon dioxide, and carbon monoxide present in cave atmospheres.”
I still have my doubts about the hydrogenotrophic nature of actinobacteria. Please also split these arguments. Primary production is performed by different microorganisms. This includes abundant and active methanotrophs. Novel lineages of Actinobacteria encode the capacity to grow as chemoautotrophs. Together, they may use trace gases such as methane, hydrogen, and carbon monoxide as energy sources."

L413: Again, you did not at all quantify the contribution of aerotrophy to the cave ecosystem. All these statements require a comparison of methane/H2, etc, consumption

L420: Better: "Altogether, our analyses suggest that atmospheric trace gases support a basal level of primary production and energy conservation, which in turn sustains the substantial biodiversity found in various cave ecosystems."

Reviewer #3

(Remarks to the Author)
Thanks to the authors for their careful and well-balanced rework of the submitted manuscript.
No further requests.

RESPONSE TO REVIEWERS

Reviewer #1 (Remarks to the Author):

Bay and coworkers strongly increased the quality of the manuscript, and I fully agree with the molecular analysis and observations made in Fig. 1 to 3. Indeed, caves harbor many microorganisms with the genomic potential to use H2, CO2, and CH4 airborne gases. But do they use this potential to produce a substantial amount of chemolithogenic biomass? I question the claims made about the importance of these substances as energy sources for microorganisms.

We thank the reviewer for this important question and agree to tone down prediction in this regard by removing subjective terms such as *high* and *significant* without qualification.

L449-453 - Methanotrophs also contribute to carbon acquisition, as they are estimated to assimilate -3.5 x 10^{-7} to - 2.0 x 1.02^{-12} nmol C $cell^{-1}$ min^{-1} based on their *ex situ* cell-specific oxidation rates, and a theoretical 40% carbon conversion efficiency, which is further supported by their activities based on the *in situ* flux analysis (Fig. 5b; Extended Data Table 7b).

L279 - Trace gas oxidation occurs together with nitrification and sulfide oxidation

We have removed the term '*significant*' where no statistical test was performed, including the following sentences:

L359-362 - Power per cell outputs were similar between rock types and cave depths for H_2 and CO, but were higher in basalt compared to limestone, and between surface and subsurface for CH_4 (Fig. 4d; Extended Data Table 5b).

L453-455 - These experiments demonstrate that microbial energy and carbon acquisition from atmospheric substrates occur continuously, with chemosynthetic primary productivity being sustained across a range of cave surfaces.

There might indeed be higher H2 concentrations at the cave's entrance than in the twilight zone, but I do not see any further trend toward the interior of the caves. If so, only the entrance /twilight zones show hydrogen consumption, but the middle and deep zones of the caves do not further contribute to hydrogen oxidation, and the same holds for other gases.

We respectfully disagree that the absence of a further declining trend of gas concentrations with the distance from the interior of the caves necessarily implies no microbial oxidation in the interior. As we explained thoroughly during the last revision, gas concentrations within caves are influenced by multiple factors especially cave geomorphological features that affect gas equilibrium. In a system where gas transport and equilibrium exceed local consumption, it is reasonable to expect little variations in measured concentrations. For example, soils are known to be the largest sink for trace gases but the high equilibrium rates of the atmosphere will mask local consumption if ambient gas concentrations at soil surface are considered as a sole indication of consumption. The distance from the twilight to deep zones within the sampled caves is relatively short. Gas diffusion and mixing is expected to occur rapidly, though a moderate declining trend of gas at the deep interior is still sometimes observed (e.g. in limestone cave).

In the manuscript, we provide multifaceted evidence that trace gas oxidation is widespread (by genome-resolved metagenomics) and active (by flux, activity, and isotope studies) across all caves samples. Active trace gas oxidation by cave samples across all depths was validated through both *in situ* flux chamber measurement and *ex situ* microcosm incubations. These rates are theoretically sufficient to maintain the energy needs of all microbes capable of using these gases, as well as generate biomass. As in the last revision, we agree with the reviewer that we cannot determine exactly what proportion of energy and carbon is generated from atmospheric energy sources compared to others, and have ensured that this is appropriately reflected in our text.

Nevertheless, we agree that the activity at entrance and twilight zones was inadequately described and have clarified this further in text. Reflecting the limitations of the static flux chamber approach, including insufficient sediments in one of the lava tubes, we used *ex situ* microcosm assays to confirm trace gas activity. *Ex situ* microcosm assays across all zones confirmed active oxidation within every section of the transect (entrance, twilight, transition, deep) in each of the four caves. Despite lower rates under potential substrate (CH_4, H_2, CO) limitation, these communities appear to fully retain their chemosynthetic activity, even when *in situ* concentrations are diminished below atmospheric.

We have changed the following:

Section: *In situ* rates

Removed the term significantly and replaced with the average fold change instead.

L289-291 - Ambient air concentrations of all three gases decreased 4-fold from the cave entrances to the interiors of the limestone caves, suggesting microbial consumption (Fig. 4a; Extended Data Fig. 7).

Section: Energy yield calculation

We added a cautionary statement, highlighting that the high energy yield samples were constrained to entrance and twilight sites in basalt caves.

L367- 369 - Nevertheless, the observed rates may theoretically provide sufficient power to support the growth and survival of localized populations of trace gas oxidisers in these caves, particularly at the entrance and twilight sites in basalt caves.

The authors make statements such as high/ substantial rates without bringing their data into context. How much is 1 nM per m² and sec or 86.4 µM per sqm and day? Let's get the number into context. The lowest oxygen fluxes on Earth were measured for the South Pacific Gyre- about 0.1 to 1mmol per sqm and day. In the South Pacific Gyre, the oxygen penetration depth is 100 meters. I don't think that anything similar can be observed in the cave.

We thank the reviewer for raising concern regarding the contextual framing of gas fluxes and subjective terminology.

We have removed the subjective term 'greatly' and amended the section as follows:

L295-298 - On average, *in situ* CH_4 fluxes increased from 1.4 nmol m^{-2} s^{-1} at the entrance to 3.1 nmol m^{-2} s^{-1} inside (mean of transient and equilibrium flux estimates), consistent with localized methanotrophic activity within caves (Fig. 4b; Extended Data Fig. 7, Extended Data Table 5a)

We agree that the term 'substantial' is too subjective and have removed it from the following sentences:

L132-135 - A smaller proportion of cave microbes can also use rock-derived energy sources such as sulfide (13.9% / 18%), thiosulfate (6.7% / 7%), ammonia (4.1% / 3.5%; Extended Data Fig. 4), nitrite (1.2% / 3.7%; Extended Data Fig. 5), and ferrous iron (2.7% / 2.2%).

L463-466 - Cave ecosystems differ from polar desert soils, the other major type of ecosystem shown to be atmospherically powered [19–21], in that trace gases appear to drive continual growth rather than long-term survival in these nutrient-deprived environments.

The oxygen consumption in this cave is likely many times higher than the contribution of CO (clearly), hydrogen (for sure in the deeper cave), and methane. Hence, airborne autotrophy will also result in only traces of biomass production, which will hardly affect the carbon flow in the cave. These are just estimates, but the key experiment to call the hydrogen consumption substantial / and, if at all, is connected to maybe 1% of the oxygen consumption (this would be my estimate) has not been done.

We thank the reviewer for this important point. We agree that without direct O_2 consumption measurements, the relative contributions of CH_4, H_2 and CO oxidation to total respiration and biomass production remain unclear. As a caution we have removed subjective terms and emphasised localized microbial activity rather than ecosystem scale inferences on carbon flow.

We have made the following changes to acknowledge the limitation of no O_2 measurements and highlighting the relative high power rates at entrance and twilight sites in basalt caves.

L362-366 - As we did not simultaneously measure oxygen consumption, we are unable to determine what proportion of total aerobic respiration is attributable to trace gas oxidation. These values represent theoretical estimates averaged across all cells, and it is likely that only a fraction of the community is metabolically active, meaning the per-cell power for active trace gas oxidisers may be higher.

L386-389 - Altogether, our data indicate that atmospheric trace gas consumption can theoretically support localized activity, including growth of the methanotrophs and the survival of the hydrogenotrophs, with some bacteria potentially capable of chemolithoautotrophic growth using H_2 and/or CO.

Because of the low rates, cell-specific reaction rates become unrealistically low (Fig 4c-d). Data points such as 10^14 nmol mean the respiration of single molecules per second. This would be difficult to replicate – it would take 7 billion seconds to fix the carbon of the average cell. It is more likely that some few cells are active, but this makes average cell-specific rates odd. It is like having 100000 homeless and Elon Musk at your party and stating, based on average, that all your guests are millionaires.

We appreciate the reviewers' important point regarding low cell specific rates and their relation to power yield required for growth. A major challenge in this field is discriminating exactly what the reviewer indicates: is atmospheric trace gas oxidation dominated by a several highly active cells (the highest values) or billions of low activity cells (the lowest values)? This is very difficult to discriminate given H_2 and typically CO are not directly assimilated into biomass, unlike CH_4, making techniques

such as SIP unreliable. It is likely somewhere in the middle. Most cells likely benefit from a low level of H_2 and CO oxidation to sustain their basal (maintenance) needs and use these gases purely as energy sources; indeed, for most cells, the generation of 10^{-13} to 10^{-15} W per cell will be sufficient for this purpose. However, it is likely that some cells are also adapted to use these gases to fix carbon dioxide as primary producers, including potentially much more quickly. We have made the following changes to the entire power per cell section to caution our interpretation of the theoretical power calculations derived from bulk cell-specific rates.

L357 - 389 - Thermodynamic calculations based on bulk oxidation rates of H_2, CO, and CH_4 revealed that trace gas oxidation rates inside the cave yielded an average power output of 1.3×10^{-15}, 8.3×10^{-16}, and 5.8×10^{-13} W per H_2, CO, and CH_4-oxidising cell, respectively. Power per cell outputs were similar between rock types and cave depths for H_2 and CO, but were higher in basalt compared to limestone, and between surface and subsurface for CH_4 (Fig. 4d; Extended Data Table 5b). As we did not simultaneously measure oxygen consumption, we are unable to determine what proportion of total aerobic respiration is attributable to trace gas oxidation. These values represent theoretical estimates averaged across all cells, and it is likely that only a fraction of the community is metabolically active, meaning the per-cell power for active trace gas oxidisers may be higher. Nevertheless, the observed rates may theoretically provide sufficient power to support the growth and survival of localized populations of trace gas oxidisers in these caves, particularly at the entrance and twilight sites in basalt caves. Indeed, based on our culture-based studies of four oligotrophic bacteria that grow on trace gases alone (e.g. *M. gorgona*), the CH_4 power output in cave greatly exceeds growth requirements and H_2 power output is at the same magnitude to support growth[42,50]. Importantly, these rates are averaged for all cells and samples, while in reality some microbes may co-consume these gases, notably the highly abundant *Hydrogenocavus* and *Hydrogenomurus*. Altogether, our data indicate that atmospheric trace gas consumption can theoretically support localized activity, including growth of the methanotrophs and the survival of the hydrogenotrophs, with some bacteria potentially capable of chemolithoautotrophic growth using H_2 and/or CO.

Because it is impossible to compare the low hydrogen, methane, CO oxidation rates with the missing total oxygen consumption rates, statements such as the last sentence of the abstract are not justified: “Trace gases sustain a basic level of biodiversity and productivity through a process we define as aerotrophy that is supplemented by organic and inorganic input”. In fact, looking at the data, it is rather unlikely that the contribution of these trace gases to the overall microbial activity is very high. A solution for this abstract could be: “The energy from airborne trace gasses may support the growth of a yet unquantified share of the cave communities. These organisms may add organic carbon compounds to the energy-limited cave ecosystems. “

We have amended the abstract according to the reviewer’s suggestion. We have made an amendment to reflect the key point that trace gases do not just support primary production in these ecosystems: they also sustain the basal energy needs of communities.

L40-43 - *In situ* and *ex situ* biogeochemical and isotopic measurements consistently confirmed that these gases are rapidly consumed at rates sufficient to meet community-wide energy needs and potentially drive primary production.

L46-49 - Consumption of atmospheric trace gases likely has a dual role in cave ecosystems, providing energy to sustain survival of diverse microbes and potentially supporting chemosynthetic growth of an undefined fraction, thereby introducing

organic carbon into the system. This process, which we herein define as 'aerotrophy', operates alongside organic and inorganic inputs.

The entire paper has an awkward tendentiousness. Whenever a rate shows a bit towards "aerotrophy" it is excessively reported: Just look at Fig. 4ab, discussed in line 250ff. Gas concentrations decreased significantly. I don't see significant value. If you start from twilight, there is simply no decline anymore. Wording like high rates do not match if you cannot compare it with other rates.

We thank the reviewer for this critical observation. We agree that terminology such as significant, high and substantial can be misleading if not qualified by statistical reference or clear reference point. We have systematically revised the manuscript to remove these subjective terms throughout, as described above.

Fig. 4. B: Fluxes are never negative according to the graph. But look at the MS. This would also be impossible because it is a logarithmic scale. Numbers of replicates are not given. But data points are up to 5 orders of magnitude different.

We thank the reviewer for this important point. All fluxes represent net uptake (negative values) and are plotted as positive values on a log scale for visualization. We have amended this in the figure caption and main text make this clear.

Fig. 5: There is much higher methane than CO2 fixation (Fig. 5 c,d) – but the d13C argues against methanotrophy and more for CBB-derived fixed carbon. Of course, most of the organic carbon is not produced by microorganisms but is from sunlight-fixed organic matter.

We thank the reviewer for their interpretation of the d13C data. We agree the data is suggestive of organic carbon from cave samples is predominantly contributed by CBB carbon fixation and it is reflected in this discussion of the results, in addition of the caution that possibility of these organic carbon being derived from external plant derived matter:

The organic fractions in the cave samples exhibited a depletion of ^{13}C ($\delta^{13}C_{organic}$) from -21.7 to -67.4 ‰, consistent with biomass derived from CBB cycle, but distinct from other carbon fixation pathways[51,52] (Fig. 5a). It is likely that some of organic carbon are external plant-derived inputs (allochthonous inputs) transported into cave interiors.

However, it's highly likely that that some organic carbon is derived from methanotrophy and microbial carbon fixation. First, both correlations and random forest analysis showed that marker genes for CBB (RuBisCO) and methanotrophy (PmoA) are the best predictors of measured d13C values, noting the former is widespread in microbes and plants. Second, biofilm samples which showed more negative d13C values of organic carbon than sediment samples are more enriched in methanotrophs. Both observations cannot be reasonably explained if organic matter of the sample is solely derived from same sunlight-fixed organic matter. We provide balanced consideration of both processes in the revised manuscript.

L244ff: I cannot agree that trace gas consumption is described as high rates.
Fig. 4a: Extended data Figure 7: I do not see a substantial or statistically significant

decrease for most curves, except “entrance” and methane in twilight. If so, there is a difference between T0 and T1 – not after. The entrance of the cave might be a difference.

We thank the reviewer for highlighting this observation in our flux data. Indeed, ED Figure 7 shows that the majority of the observed flux for H_2, in particular at entrance and twilight samples stem from the t0-t1 step.

For context, we have repeated the calculations and now present a dual flux analysis, where we show a ‘transient flux phase’, which includes the t0 timepoint, and an ‘equilibrium flux phase’, which shows the fluxes under equilibrated conditions, excluding t0. Transient flux measurements are therefore a high range estimate, since they include the full time series with ambient conditions. They capture the maximum potential uptake rate, incorporating the steepest concentration drop (ambient (t0) - t1). They are highly sensitive to changes in initial concentration and therefore have the potential to overestimate steady state consumption. Equilibrium fluxes are predicted to be a lower range estimate since they only capture consumption once the chamber conditions are fully equilibrated. Removing the ambient condition and exaggerated initial draw down, equilibrium fluxes are a more conservative lower bound estimate on microbial soil to air fluxes.

By presenting both transient and equilibrium fluxes side by side (Fig. 4b), we show the likely maximum (transient) and minimum (equilibrium) bounds of microbial gas uptake. This is also in light of the large flux range, which covers multiple orders of magnitude. Both analyses show net *in situ* consumption of all gases, albeit at varying rates, supporting our conclusions.

Overall, this analysis shows that H_2 fluxes at entrance and twilight zones are mostly affected by the rapid change between t0-t1 timepoint. Carbon monoxide and methane fluxes, largely retain their flux trend across all samples under both transient and equilibrium flux phases, and retain an increasing trend with cave depth, in line with our conclusions.

Line 343ff: particularly high hydrogenotrophic carbon fixation rates of 6.7*10^-12 nmol per cell and minute – that does not sound to be a lot.

We thank the reviewer and have toned down this statement.

L418 – 422 - Notably, basalt biofilm and soil microbes mediated higher hydrogenotrophic CO_2 fixation activities (4×10^{-13} nmol ^{14}C cell^{-1} min^{-1}) compared to average rates by limestone sediment microbes (5.8×10^{-14} nmol ^{14}C cell^{-1} min^{-1}). This supports our metagenomic inferences that H_2 is the predominant driver of CO_2 fixation and may contribute to the observed $\delta^{13}C_{organic}$ signatures in caves.

It is unclear what a strong consumption means; see comment above. A change of a fractions of a ppm difference in flux chambers is never a lot. Here and in other cases, I miss comparison with other habitats / or other rates.

We agree that the term ‘strong’ is too subjective without direct comparison and have removed it through the manuscript.

Line 364: About half of the carbon should come from CO2 when methane is assimilated. Methane assimilation cannot be three orders of magnitude higher than CO2 assimilation. You may have triggered methane oxidation by the addition of large amounts of 14C-tracer.

Unfortunately, methods do not describe 14C-methane oxidation rates. I fear that these high rates derive from incubations with large amounts of 14C: 1 MBq carbon/CH4 is already 0.4 µmol. Please report also on the duration of this incubation.

We thank the reviewer and would like to clarify our approach. No ^{14}C-labelled methane was used in this study. All radiotracer experiments were conducted using ^{14}C-bicarbonate to measure CO_2 fixation under H_2 enriched conditions. We estimated methane carbon assimilation based on our *ex situ* microcosm experiments and the derived cell specific reaction rates. We applied a 40% carbon conversion efficiency based on literature values to determine a theoretical approximation. These values were not intended to serve as a direct comparison to our ^{14}C CO_2 fixation rates.

We have clarified this in the text.

L449-453 - Methanotrophs also contribute to carbon acquisition, as they are estimated to assimilate -3.5 x 10^{-7} to - 2.0 x 1.02^{-12} nmol C $cell^{-1}$ min^{-1} based on their *ex situ* cell-specific oxidation rates, and a theoretical 40% carbon conversion efficiency, which is further supported by their activities based on the *in situ* flux analysis (Fig. 5b; Extended Data Table 7b).

14CO2-based rate measurement: (line 637) 14C isotope labeling is not the correct terminology here). Note that you measure hydrogen-dependent CO2 fixation rates at H2 concentrations that are 100 times elevated compared to nature. Still, these rates are small.

We thank the reviewer and agree with both points. We have revised the terminology to $^{14}CO_2$ fixation assay throughout. We also agree that hydrogenotrophic CO_2 fixation assays don't represent hydrogenotrophic CO_2 fixation at ambient H_2 concentrations. Instead, enriching the H_2 concentration was necessary to elicit a measurable response above background $^{14}CO_2$ fixation. This also reflects competition from unlabelled bicarbonate and internal CO_2 cycling. We have added this caution to the text, emphasising these findings as a conservative estimation of CO_2 fixation potential rather than absolute values.

L422-427 - Gross carbon fixation rates are probably orders of magnitude higher than measured $^{14}CO_2$-fixation rates, as unlabelled native inorganic carbon and internally recycled CO_2 within samples invariably competed with the trace levels of added $^{14}CO_2$, especially in carbonate-rich limestone samples. Therefore, these rates are a conservative estimate to only reflect the potential for hydrogenotrophic CO_2 fixation under elevated H_2 conditions, not ambient rates.

Extended Fig. 8: Here, a logarithmic curve is not good - or tells a lot – the organisms only start picking on the higher concentrations after a lack phase. This may suggest that environmental trace gas concentrations are hardly used.

We agree with the reviewer that as a standalone figure it is not meaningful. We added Extended Data Figure 9 to show the oxidation traces as well as linear and nonlinear model fits.

Please find a meaningful order of the methods i.e., best use the order of how data appears in the MS

Thank you, we have amended the order of methods to follow the manuscript flow.

Reviewer #2 (Remarks to the Author):

The authors have addressed nearly all of my concerns, and the manuscript is much improved. I still don't like "aerotrophy" as used here, but I seem to be alone in this opinion.

We thank the author for their review.

Reviewer #3 (Remarks to the Author):

Comments and Suggestions for Authors

Bay and colleagues have investigated metagenomic capacities and actual biogeochemical cues for active microbial consumption of atmospheric trace gasses, mostly H2, CH4 and CO, in 4 Southern Australia cave systems. Cave microbiota are compared across longitudinal transects and different compartments of two basalt and two limestone caves in Victoria. With ~1500 high-quality MAGs assembled for many dozens of samples, ~half of the MAGs hosted capacities to consume either one of the different trace gasses. Amongst them, abundant hydrogenotrophic candidate genera within the Actinobacteriota, methanotrophic gammaproteobacterial Ca. Methylocavales, and also some MAGs of enigmatic candidate phyla. Aerotrophic primary production is shown to be substantial throughout most samples, with methane and hydrogen-derived energy delivering enough power-per-cell to allow for continued growth, or at least sustained activities and survival in lack of other substrates. The concept of, at least temporary or partial, aerotrophy as a dominating physiology in caves is a highly interesting addition to our understanding of these underexplored ecosystems. This piece clearly warrants publication in a journal like NatComms.

Still, before acceptance, the authors should address the following comments:

1. It Would be good if the results (& discussion) section could start with some basic primers on the nature of the sampled cave systems (basalt and limestone), locations (entrance, longitudinal transects) sample types (biofilms, sediment, …) and overall sample numbers. This is important to grasp the scope of the study, as not every reader will (first or at all?) grind through the M&M or supplement. Just a bit more info on the basic settings of the study, before delving into all the community composition and the metagenomic details.

We thank the reviewer and agree to add an additional set up to this section. We have added the following text:

L98-102 - We studied four aerated caves in southeastern Australia, two limestone and two basalt lava tubes (Fig.1a). In each cave, we sampled along a transect extending from the entrance to twilight, transition and deep zones. We collected a total of 94 environmental samples including sediments, biofilm, and mineral samples, which were analysed using metagenomics as well as gas and isotopic studies to determine microbial composition, function and metabolic activity.

2. In contrast to the authors notion, caves do typically not contain soil (Fig. 1b). Unless it is washed in or collapses in from above. Please change to “Sediments” in Fig. 1b, as correctly implemented already in other Figs. Also, “soil” must still be replaced on some occasions throughout the text, e.g. L129 or L343.

We thank the reviewer and agree that in the absence of a true soil matrix ‘sediment’ is a more appropriate term. An exception are samples collected at the cave entrance or doline where the material on the cave floor reflected soils rather than sediment. To

ensure consistency we have changed the name from soil to sediment in all instances, which exception for entrance samples, referring explicitly to material resembling characteristics of surface soil.

3. L318: this must be a mistake. Explain that this was just one sample of many dozens with this extremely light signature at MINUS (!) 67.4 permil. And that this is much too depleted to be consistent with the CBB cycle OR any typical methanotrophs growing on light methane. This must point towards a local influx of geogenic, isotopically highly depleted (potentially fossil ?) methane in this one limestone sample. But I agree that especially the biofilm samples in basalt (lighter than -30 permil) are in clear agreement with the CBB

We thank the reviewer for the insightful comment. The one sample with highly negative $d^{13}C_{organic}$ values was not sequenced and thus we could not confirm the main pathway of organic carbon formation that best explain the measured value. As a caution, we have now elected to remove this outliner and clarified this in the methods.

4. There is a clear increase of methanotrophs towards the cave interior. Same as for methane (Esp. for basalt), consumption rates, fluxes, and per-cell power availability. All of this is not fully consistent with a consumption (and influx) of atmospheric methane via the entrance alone. In addition, there must be a contribution of geogenic methane influx. This should be better discussed, same as its implications for the “aerotrophy” concept as defined by the authors. Geogenic methane is still a trace gas, but does not come from the atmosphere.

We thank the reviewer for this important point. A limitation of our study is that we did not use isotope studies to differentiate the sources of atmospheric trace gases. We amended the manuscript as follows:

L258-261 - It is likely that these gases are predominantly atmospherically derived, given their concentrations are at or below global average concentrations of these gases in the lower troposphere[47–49], though it can’t be ruled out that they are also produced through geogenic and biological processes and maintained at such concentrations through microbial consumption.

L470-473 - Given the relatively stable environment, humid conditions, and aeration of most caves, it is predicted that aerotrophs will continually cycle gases such as hydrogen, methane, and carbon dioxide, whether of atmospheric origin or geogenic origin (including at atmospheric concentrations), allowing continuous primary production.

5. Along the same line, I am perfectly fine with the authors’ findings on aerotrophic chemosynthesis being ubiquitous and unexpectedly important in cave microbiota. Their overarching conclusions on aerotrophy being able to sustain microbial life clearly beyond mere survival or maintenance energy needs and over longer time spans are convincing and novel. But still, I suggest to add another grain of caution to some remaining statements on e.g. “dominant primary producers” e.g. line 153. More sampling and data (seasonal or hydraulic event-triggered) would be required to really waterproof such posits, especially for members of the well-known and typically heterotrophic lineage of the Actinobacteriota. At least the option of transient and dynamic heterotrophic, mixotrophic, or aerotrophic growth should not be excluded.

We thank the reviewer and have revised as follows:

L174-179 - Most hydrogenases, CO dehydrogenases, and RuBisCOs were co-encoded by the most abundant Actinobacteriota lineages residing in the caves (primarily classes Actinomycetia, Thermoleophilia, Acidimicrobiia, and *Ca*. Aridivitia)[21] (Fig. 3a), suggesting that they may be important primary producers in these ecosystems, although we cannot exclude the possibility of transient and dynamic heterotrophic and mixotrophic strategies.

Also the abstract does not offer any conditional or cautioning statements in the latest version. Please add accordingly.

We agree and note that a similar point was raised by Reviewer 1. We have amended the final sentence of the abstract as follows:

Consumption of atmospheric trace gases likely has a dual role in cave ecosystems, providing energy to sustain survival of diverse microbes and potentially supporting chemosynthetic growth of an undefined fraction, thereby introducing organic carbon into the system. This process, which we herein define as 'aerotrophy', operates alongside organic and inorganic inputs.

Minor comments:
L97: Consistent taxonomic rigour must be used throughout MS, Text and Figs. E.g. Proteobacteria are correctly named "Pseudomonatdota" etc. Please check https://doi.org/10.1099/ijsem.0.005056 and elsewhere for the latest authoritative nomenclature.

Our manuscript is taxonomically rigorous and adopts the taxonomy of the GTDB release (GTDB release 214, April 28 2023) in which we annotated these MAGs.

L103: marker genes of basic physiologies?

We thank the reviewer and have clarified as follows (using key rather than basic):

L122-124 - The energy and carbon acquisition strategies of the cave microbes were inferred by searching for 52 conserved marker genes encoding the potential of key energy and carbon acquisition processes in the MAGs and short reads (Fig 2a; Extended Data Table 4a-c).

L116: give abundance range of photosynthesis genes at entrance.

We thank the reviewer and have added this as follows:

L161-L162 - As expected, photosynthesis genes were abundant in sediments and biofilms at the entrance of each cave (29%) but declined by an average of 72-fold in the cave interior.

L139: I would refrain from calling them "strategies", as long as overall activity and rate data are not always available. "Capacities"?

We thank the reviewer and have added the term 'potential' as follows:

L122-124 - The energy and carbon acquisition strategies of the cave microbes were inferred by searching for 52 conserved marker genes encoding the potential of key

energy and carbon acquisition processes in the MAGs and short reads (Fig 2a; Extended Data Table 4a-c).

L167: Pseudomonadaceae are NOT within the Actinomycetia, no way! Egibacteracerae, of course, are?

We thank the reviewer for spotting this unfortunate error. We have corrected this to *Pseudonocardiaceae* which are Actinomycetia.

L169: caves appear to…

We thank the reviewer and have checked this sentence. It reads: Thus, caves appear to select for highly productive actinobacterial primary producers that grow on atmospheric energy and carbon sources.

L 171 here and elsewhere report findings in past tense.

We thank the reviewer and have checked the manuscript for these instances throughout.

We thank reviewers 1-3 for their insightful and thorough and thoughtful comments. We hope these edits address their concerns and present a more precise and balanced narrative.

AUTHOR RESPONSE

Reviewer #1 (Remarks to the Author):

Bay and coworkers significantly improved the paper; however, I still have some questions/ remarks:

We thank the reviewer for their further appraisals and suggestions, and have further toned down and cautioned our findings.

L41ff: at rates sufficient to meet community-wide energy needs this need to be tested differently, as it would mean that all biomass would cycle internally, i.e. also biomass of heterotrophs. Below I used your data from CO2 fixation that this is not the case. i.e. in a single example I calculated biomass turnover time of 35,000 years. I simply doubt that this meets the energy demands of all organisms. Maybe: with rates which likely sustain the energy demands of a substantial fraction of the community."

The abstract has been revised accordingly.

L114: keep consistently present tense when talking about metagenomic data, “encode”, and past when you talk about rates...

We agree and have revised throughout.

L120: be moderate: You can not infer from MAGs that organisms do something: … of cave microorganism that encode enzymes that would allow the use of.. or something

We agree and have tempered claims of function as follows:

L121: These findings suggest that most microbial cells in caves encode enzymes that could enable trace gas oxidation.

L153: avoid novel here

We have replaced with ‘previously uncharacterised’ to reflect these microbes have not been previously characterized genomically and metabolically.

L169: belong to high-affinity clades of hydrogenases

We have revised this sentence accordingly:

L177-180: Corroborated by the short-read analysis (Fig. 2a, Extended Data Table 4b), almost all of these belong to high-affinity clades of hydrogenases (groups 1h, 1l, and 2a [NiFe]-hydrogenases[21,39,40]) (Fig. 2a), suggesting they are adapted to atmospheric rather than elevated levels of these gases.

L183: I discourage the authors from naming uncultured organisms after potential energy sources. i.e., to my knowledge, there are no other hydrogenotrophs within the Pseudonocardinaceae, Egibacteraceae, and Actinomycetia – all of them are rather heterotrophs. Spreading this attribute and manifesting it in their names, based on metagenomic information, seems to be wrong. To my knowledge there are no knallgas bacteria in the Actions. Culture them in the lab, investigate their capabilities, and then this naming is adequate. Instead you may use locations in the name.

We consulted with the Editor on this point and they have confirmed they are happy for us to use the originally proposed names. We respectfully maintain that function-based candidate names are appropriate under current Candidatus nomenclature conventions and in harmony with the SeqCode and GTDB placeholder naming logic.

Autotrophic growth on H_2 has been reported on multiple actinobacterial isolates, including reports from over 60 years ago, such as *Pseudonocardia autotrophica* (previously known as *Streptomyces autotrophicus*; doi.org/10.1007/BF00424890) and *Mycobacterium gordonae* (doi.org/10.1099/00207713-24-3-338). These actinobacteria similarly encode the same set of genes required for Knallgas-like metabolism as we identified in the three named cave actinobacterial taxa (group 1h hydrogenases and CBB cycle, in addition to CODH).

Our proposed names are based on high quality MAGs (completeness ≥ 84 %, contamination ≤ 3.3 %) underscoring robustness of their metabolic predictions.

- **'*Candidatus* Hydrogenomurus basanatii' (MAG SE1519_metabat2.bin.2; 100% / 2.8%)**
- **'*Candidatus* Hydrogenocavus calcicola' (MAG M3-B2_metabat2.bin.58; 96.6% / 3.3%)**
- **'*Candidatus* Hydrogenolapis speleophilus' (MAG SD8021_metabat2.bin.4; 84.1% / 1.1%)**

The 2019 International Code of Nomenclature of Prokaryotes (Parker *et al.* 2019) explicitly allows genome assemblies combined with predicted metabolic features and habitat preference to underpin a *Candidatus* designation (Appendix 11). Guidance on the vernacular epithet (Appendix 11.3) states that it may be any genus / species names provided the description include genomic, physiological / metabolic and environmental information.

This approach has been widely adopted, prominent examples include '*Candidatus* Methanoperedens nitroreducens' (Haroon *et al.*, 2013, *Nature*), named for its nitrate-dependent methane oxidation, and '*Candidatus* Nitrososphaera gargensis' (Spang *et al.* 2014, *Environmental Microbiology*), named for its ammonia-oxidizing capacity. These function-based names are both permissible under the ICNP guidance and remain highly informative for the wider research community.

Our etymologies in Supplementary note 2 include MAG source, quality metrics and linguistic derivations. For example, *Hydrogenomurus basanatii* combines N.L. *hydrogenum* ("hydrogen") + L. *murus* ("wall"), with *basanatii* denoting "of the basalt cave." Overall names accurately reflect the predicted trace gas lifestyles and are derived from high quality genomic data and robust functional gene predictions.

L293: Sulfur and iron are only energy substrates when they are reduced. Total S and Fe are not important. What species do you speak of?

We thank the reviewer for highlighting the need to specify redox species. We report total sediment ammonium, sulfur etc., only for geochemical context. The consumption of these substrates was determined by our nitrification and sulfide oxidation assays, which measured changes in the aqueous species NH_4^+, NO_2^-, NO_3^-, and SO_4^{2} respectively as reported in Extended Data Table 6. We have modified the text to better clarify this.

L321-325: "The cave sediments contained varying concentrations of ammonium (1.35-40.2 mg/kg), sulfur (7.3-2468 mg/kg), and iron (6.96-2003 mg/kg) (Extended Data Table

1). Microcosm incubations showed ammonium was oxidised at variable rates across the samples and increased from the entrance to interior of the cave (Fig. 4e; Extended Data Fig. 11; Extended Data Table 6).”

L323: Again, I do not see convincing evidence for the growth of these organisms as hydrogen oxidizers. If you want to state this, cultivate the organisms.

As we elaborated in the text, the inference is based on multiple independent evidence, including the genomic characterization of these organisms, carbon stable isotope signatures of pure culture benchmarks of hydrogenotrophs (*C. necator* H16; *H. pseudoflava*) compared to cave samples, H_2 oxidation rate power calculation (1.5×10^{-15} W) relative to growth energy requirement of known and experimentally characterized aerotrophs (e.g. *Methylocapsa gorgana*), and the detection of stimulated $^{14}CO_2$ incorporation when supplied with H_2.

However, we agree cultivation will be necessary to definitely prove this and have now included a new sentence to better caution this:

L366-367: “We note that cultivation of these microorganisms will be a necessary next step to validate their capacity to grow on atmospheric gases.”

L 329ff: The isotopic signatures of most organic matter from cave samples are also consistent with photosynthetic matter, which is no surprise because they use the same C-fixation pathway. Only the basalt biofilm shows robust signs for another carbon source/pathway, which could indeed be methane-derived organic matter.

We have now explicitly presented this other possibility in the setup sentence of this section as follows:

L374-375: “It is likely that organic carbon from external plant-derived inputs (allochthonous inputs) is transported into cave interiors. However, despite $\delta^{13}C_{organic}$ values overlapping those of C_3-plant detritus (a shared CBB signature), multiple lines of evidence suggest cave-dwelling primary producers are also major sources of organic matter (autochthonous inputs)”.

L357ff: Please contextualize your data – here the reported carbon fixation rates: “Notably, basalt biofilm and sediment microbes mediated higher hydrogenotrophic CO2 fixation activities (5.7 × 10-13 nmol 14C cell-1 min-1) compared to average rates by limestone sediment microbes (5.3 × 10-14 nmol 14C cell-1 min-1). “Is this a lot? Let’s see how long it would take to build 1.2 % carbon - (12 mg=1 mmolC) this way, 5.3x10^-14 nmol/min per cell * 10^9 cells per g (I guess it is less in this particulate sample – we have 5.3x10^-5 nmol per minute and ccm. How much time does it take to fix 1 mM? To fix 1 mmol of carbon, this takes 1/(5.3*10-5*10-^-6) minutes = 1/(5+10^-11) minutes = 35,870 years! Therefore, it would take 35,000 years to rebuild the 1% carbon. Although sedimentation rates of organic matter in the caves will also be quite low, it appears that the contribution of CO2 fixation is relatively low. I ask for similar comments like this in earlier versions.

We appreciate the reviewer for their further comments and calculations on the ^{14}C fixation assay. However, as we have elaborated and discussed in the last revision, the $^{14}CO_2$ measurements do not reflect gross carbon fixation rates as abundant carbon (predominantly non-radioactive ^{12}C) already present within cave samples and CO_2 in the ambient air headspace will compete with added $^{14}CO_2$ (400 ppm), leading to a substantial underestimate of true carbon fixation rates. As a context, the cave samples have carbon content between 1.7 - 29%, which are many orders of magnitude

higher than the provided $^{14}CO_2$ tracer. Therefore, the multi-thousand years carbon turnover calculation by the reviewer is impropriate in this context.

The ^{14}C measurements clearly demonstrated H_2 supplementation significantly enhanced carbon fixation compared to the dark control, strongly suggesting H_2 oxidation is coupled to carbon fixation in line with metagenomic and genomic inference, whereas photosynthetic carbon fixation is minimal at inner caves.
We have now further elaborated this:

L402-406: "Gross carbon fixation rates are probably orders of magnitude higher than measured $^{14}CO_2$-fixation rates, as unlabelled native inorganic carbon, CO_2 from the ambient air headspace, and internally recycled CO_2 within samples invariably competed with the trace levels of added $^{14}CO_2$ (400 ppm), especially in carbonate-rich limestone samples (1.7 - 29% carbon; Extended Data Table 1). Therefore, these rates are a conservative estimate to only reflect the potential for hydrogenotrophic CO_2 fixation under elevated H_2 conditions, and should not be interpreted as ambient net CO_2 fixation rates."

Since methanotrophs will always fix ~20% of the carbon from CO2, methane carbon fixation can only be four times higher than that. Additionally, we also miss discussing heterotrophic CO2 fixation, which can also be part of the discussion.

We thank the reviewer for raising this point. We would like to clarify that our methane carbon assimilation studies are based on the assumption of methane carbon assimilation primarily via the serine and RuMP pathways, not autotrophic CO_2 fixation. Soil tracer studies (Roslev et al. 1997 & 1999; Holmes et al. 1999) show that methane carbon assimilation fully encompass our 40% conversion factor. (methane oxidation rate * 0.4). We have amended this section including relevant references as follows:

L408-411: Methanotrophs also contribute to carbon acquisition from methane, as they are estimated to assimilate 3.5 x 10^{-7} to - 1.1 x 10^{-12} nmol C cell^{-1} min^{-1} based on their *ex situ* cell-specific oxidation rates, and a theoretical 40% methane-carbon conversion efficiency[53–55], which is further supported by their activities based on the *in situ* flux analysis (Fig. 5b; Extended Data Table 7b). We acknowledge that methanotrophic CO_2 fixation via the CBB cycle as well as other dark and anaplerotic processes not accounted for in this study may contribute to inorganic CO_2 fixation in caves.

In total it seems that methane oxidation rates are orders of magnitude higher than H2 oxidation (although so many more organisms encode hydrogen oxidation).

That's incorrect, the average rates of H_2, CH_4, and CO oxidation in cave samples are 6 × 10^{-2} , 8.1 × 10^{-3} , 2.5 × 10^{-3}, at their respective atmospheric concentrations (Extended Data Table 6). However, normalising these rates to a per cell basis, methanotroph cells appear to consume gases at a comparable or higher rates to the other gases (Fig. 4C).

We have amended the information provided in Extended Data Table 6 to include the intermediate steps for these calculations.

The conclusions are not justified.
L376: "Here we provide extensive metagenomic and biogeochemical evidence that diverse caves are atmospherically powered ecosystems." See above. Given your data (i.e., H2 consumption, CO2 fixation), I don't think you can make this claim. As suggested earlier, and implemented before: Here we provide extensive metagenomic and biogeochemical evidence

that diverse caves are atmospherically powered ecosystems.
Here, we provide metagenomic and biogeochemical evidence for the consumption of atmosphere-derived trace gases in cave sediments and biofilms (note that this does not include lakes, walls, etc.). This consumption may feed a yet-to-be-quantified fraction of the microbial community. (or something similar).

We have toned down the statement and amended the sentences as follows:

L421-423: Here we provide extensive metagenomic and biogeochemical evidence for the consumption of atmosphere-derived trace gases in cave sediments and biofilms. This consumption may support a potentially large fraction of the microbial community.

“Primary production appears to be driven by highly abundant and active methanotrophs, as well as novel lineages of actinobacterial lithoautotrophs, that continuously use the gases methane, hydrogen, carbon dioxide, and carbon monoxide present in cave atmospheres.” I still have my doubts about the hydrogenotrophic nature of actinobacteria. Please also split these arguments. Primary production is performed by different microorganisms. This includes abundant and active methanotrophs. Novel lineages of Actinobacteria encode the capacity to grow as chemoautotrophs. Together, they may use trace gases such as methane, hydrogen, and carbon monoxide as energy sources."

We thank the reviewer for the suggestions and have split these arguments as follows:

L423-424: Primary production appears to be driven by highly abundant and active methanotrophs oxidising atmospheric methane. Novel lineages of actinobacterial lithoautotrophs are predicted to oxidise hydrogen to fix CO_2. Together, these organisms continuously use the gases methane, hydrogen carbon dioxide and carbon monoxide present in cave atmospheres.

L413: Again, you did not at all quantify the contribution of aerotrophy to the cave ecosystem. All these statements require a comparison of methane/H2, etc, consumption

We have further cautioned as follows:

L464-465: Our analyses also do not differentiate the relative contribution of aerotrophy and other chemosynthetic carbon fixation (autochthonous inputs) and external organic matter (allochthonous inputs) to the organic carbon levels observed in the sampled caves.

L420: Better: "Altogether, our analyses suggest that atmospheric trace gases support a basal level of primary production and energy conservation, which in turn sustains the substantial biodiversity found in various cave ecosystems.

We thank the reviewer and have amended as suggested:

L482-485: Altogether, our analyses suggest that atmospheric trace gases support a basal level of primary production and energy conservation, which in turn sustains the substantial biodiversity found in various cave ecosystems.

Reviewer #3 (Remarks to the Author):

Thanks to the authors for their careful and well-balanced rework of the submitted manuscript.
No further requests.

We thank the reviewer for their time and insightful comments to improve the quality of this manuscript.