General

This study investigates the effect of bioamendments and heat stress in two soil types on microbial respiration and carbon use efficiency. This is a very relevant topic and fits well the scope of SOIL. I have, however, strong reservations about the quality of the study.

Response: We appreciate the time and the effort Reviewer#2 invested in the review our study, and we hope to have improved the quality of our work in our new version.

Reviewer: Overall, I find the introduction and methods hard to read. The introduction is overall a bit confused and does not clearly frame the interplay between heat stress and amendments in affecting the carbon cycle.

Response: Many thanks for your valuable comment. We have modified the Introduction and Material and Methods sections to improve their clarity, to be more appropriate for the research subject and conclusions. Please, see our responses to the next questions / comments and also our line-by-line responses.

Also, the relationship between bioamendments and carbon cycling was added in the Introduction section:

"Bioamendments may improve agricultural productivity by modifying soil microbial community composition and activity (Kok et al., 2023), enhancing extracellular enzymatic production for nutrient utilisation and microbial acclimation (Conant et al., 2011), providing energy and essential nutrients for soil microorganisms (C, N and P; Wang and Kuyakov, 2023), and influencing soil biogeochemistry (Mooshammer et al., 2017). They also promote improvements in soil structure and reduce soil bulk density, which could result in plant growth enhancement. Although previous research has evaluated microbial CUE under extreme heat scenarios (e.g., Dang et al., 2024; Zhang et al., 2022, Adingo et al., 2021), there is a critical knowledge gap in evaluating the impact of bioamendments application to the soil on microbial CUE under severe climatic conditions as extreme heat waves, such as those documented in Mediterranean areas. This information is needed to design holistic strategies that include the use and potential benefits of bioamendments in Mediterranean regions with challenging soil properties (low organic matter content and reduced availability of P) that are subjected to extreme heat-stress events.»

Please, see the new version of the manuscript where all the changes are shown (especially in the version with track changes).

Reviewer: there is no clear or accurate description of what CUE is, how it is conceptualised across scales and also methods, and finally more precisely how it contributes to ecosystem C cycling.

Response: We appreciate your comment. We have modified our manuscript and included your suggestions in the Introduction section, paying special attention to the definition and the role of CUE and how it directly affects ecosystem C cycling according to this suggestion:

"The alteration in soil microbial communities exposed to extreme heat stress often induces perturbation in organic matter mineralisation and C sequestration. Moreover, previous studies (Yang et al., 2023; Beugnon et al., 2025) have shown that heat stress reduces soil microbial C use efficiency (CUE; the proportion of C assimilated by soil microorganisms and allocated to biomass production rather than released as CO_2 through soil respiration), while simultaneously increasing respiration rates. This, in turn, can enhance organic matter mineralisation. In addition, soil C stocks depletion could dramatically occur under this situation if the soil does not receive sufficient C inputs. Microbial CUE plays a key role in soil C retention, soil organic C (SOC) storage and its global spatial variation, since it represents a dual microbial control point over both SOC accumulation (via biomass production) and SOC loss (via respiration; Tao et al., 2023). Therefore, microbial CUE is a more meaningful indicator of soil microbial functioning and C dynamics than respiration alone (Allison et al., 2010; Ghee et al., 2013; Li et al., 2019; Mganga et al., 2022). Additionally, soil C sequestration and CO_2 released by soil microbes are directly affected by soil nutrients content according to Kirby et al. (2014). Moreover, other research support that microbial CUE strongly influences soil C sequestration and is sensitive to various biotic (e.g., competition between species) and abiotic (e.g., pH, temperature) factors (Iven et al., 2023; Tao et al., 2023; Jones et al., 2019) . However, there is a gap of knowledge related to soil management strategies (bioamendments application) that may enhance the resilience of semi-arid soils to extreme heat-stress events (> 40 °C) to prevent widespread soil degradation (Ferreira et al., 2022). »

Reviewer: The method section is also overall unclear because some elements are mentioned in passing before being explained. Quite a few methodological details are missing (as listed in the detailed comments below), including the calculation method for CUE.

Response: The Material and Methods section was improved to be clearer and easier for reproduction (see our responses to your line-by-line comments). We have included in supplementary file figure S4, which is a schematic overview of the experimental design to explain the used methods as clearer as possible. Also, the calculation of CUE has been added for more clarity.

"Additionally, microbial immobilisation of the ^{14}C-substrate ($^{14}C_{imm}$) after the monitoring period was estimated as follows:

$$^{14}C_{imm} = {}^{14}C_{tot} - {}^{14}C_{NaCl} - {}^{14}CO_{0-t\ days} \qquad (1)$$

where $^{14}C_{tot}$ is the total amount of ^{14}C-substrate added to the soil, $^{14}C_{NaCl}$ is the amount of ^{14}C recovered from the soil in the 1 M NaCl extracts at the end of the experiments and $^{14}CO_{0-t\ days}$ is the total amount of ^{14}C recovered as $^{14}CO_2$ during the experiments (21-27 days). Then, microbial CUE for the ^{14}C substrate was estimated as follows following Jones et al. (2018a,b):

$$microbial\ CUE = {}^{14}C_{imm} / ({}^{14}C_{imm} + {}^{14}CO_{0-t\ days}) \qquad (2)\ »$$

Reviewer: Most importantly, I have concerns about the validity of the method used. The choice of 14C glucose addition is interesting here as a standardised assay to quantify CUE, since the treatments include amendments containing carbon in different forms (different C:N), whose incorporation into microbial biomass probably differ from that of glucose. This choice could potentially be justified, but it needs careful explanation and a clear description of what can be concluded about the c cycle from it in the context of this study with different amendments, both in introduction and discussion.

Response: We thank Reviewer#2 for highlighting this important methodological point. We agree that the bioamendments used in our study contain carbon in complex and heterogeneous forms (different C:N ratios, chemical structures, and decomposition rates), which would be assimilated differently into microbial biomass compared to a simple substrate like glucose. Our rationale for using ^{14}C-labelled glucose was to provide a standardised, labile carbon source across all treatments and soil types, in order to assess microbial CUE under different thermal stress conditions. By using the same substrate (glucose), we were able to directly compare microbial C allocation strategies (respiration vs. assimilation) across soils and amendments, without confounding effects from differences in the intrinsic quality or bioavailability of amendment-derived C. This approach follows established protocols for assessing microbial CUE under controlled conditions (e.g., Jones et al., 2019; Glanville et al., 2016; Sánchez-Rodríguez et al., 2024). Thus, while our results cannot be taken to describe the complete fate of amendment-derived carbon, they do provide valuable insights into how bioamendments influence microbial resilience and efficiency under extreme heat stress when microbes are supplied with labile carbon.

In the literature there are multiple references in which this method is used. We have added a few of them:

Jones, D.L., Olivera-Ardid, S., Klumpp, E., Knief, C., Hill, P.W., Lehndorff, E., Bol, R., 2018. Moisture activation and carbon use efficiency of soil microbial communities along an aridity gradient in the Atacama Desert. Soil Biology and Biochemistry 117, 68–71. https://doi.org/10.1016/j.soilbio.2017.10.026

Jones, D. L., Cooledge, E. C., Hoyle, F. C., Griffiths, R. I., and Murphy, D. V.: pH and exchangeable aluminumaluminium are major regulators of microbial energy flow and carbon use efficiency in soil microbial communities, Soil Biol. Biochem., 138, doi:10.1016/j.soilbio.2019.107584, 2019.

Sánchez-Rodríguez, A.R., del Campillo, M.C., Torrent, J., Cooledge, E.C., Chadwick, D.R., Jones, D.L., 2024. Phosphorus fertilization promotes carbon cycling and negatively affects microbial carbon use efficiency in agricultural soils: Laboratory incubation experiments. Geoderma 450, 117038. https://doi.org/10.1016/j.geoderma.2024.117038

Sánchez-Rodríguez, A.R., Del Campillo, M.C., Torrent, J., Jones, D.L., 2014. Organic acids alleviate iron chlorosis in chickpea grown on two p-fertilized soils. J. Soil Sci. Plant Nutr. 35–46. https://doi.org/10.4067/S0718-95162014005000024

Glanville, H. C., Hill, P. W., Schnepf, A., Oburger, E., and Jones, D. L.: Combined use of empirical data and mathematical modelling to better estimate the microbial turnover of isotopically labelled carbon substrates in soil. Soil Biology and Biochemistry 94, 154-168, doi:https://doi.org/10.1016/j.soilbio.2015.11.016, 2016.

Reviewer: If 14C glucose as a general method could be, perhaps, justified, different incubation times for different treatments constitutes a methodological bias. It is clearly stated that different treatments were subject to different incubation times. Incubation time (after which 14C remaining into the soil was measured, which I assume was used to estimate incorporation of 14C into microbial biomass) appears to be based on the time it takes for CO2 emission rates to stabilised, which expectedly differed between temperature treatments. In my sense, this does not allow comparison of CUE in the different temperature treatments, thus providing a biased method to address the key question of understanding the impact of heat stress on CUE. This is because incubation time in substrate incorporation methods to calculate CUE determines largely how CUE can be conceptualised, with increasing incubation time increasing the chances of added inputs being exuded, turned-over or maintenance respiration, rather than contributing to growth. If not accounted for, these processes can lead to overestimations of the fraction of substrate assimilated into from the classical equation:

CUE = 14C biomass / (14C biomass + 14 respired).

Response: We appreciate the reviewer's insightful comment regarding incubation time. We agree that incubation time is a critical factor influencing microbial CUE because longer durations may incorporate processes beyond initial assimilation (e.g., turnover, maintenance respiration).

Our approach was guided to capture complete respiration dynamics after 14C glucose addition. Microbial respiration was stabilised at different times depending on the temperature. We, therefore, extended incubations until $^{14}CO_2$ release plateaued for each temperature, following established protocols (Jones et al., 2019; Glanville et al., 2016). This ensured that cumulative CO_2 release reflected the full mineralisation of added glucose under each thermal regime with the different bioamendments. Regarding the comparability across soils/treatments. If a fixed length of monitoring of the experiment had been applied (e.g., 21 or 27 days), the faster dynamics at high temperature would have been truncated, underestimating respiration and overestimating CUE relative to lower temperatures (more details at line-by-line answers below). This only happened for Experiment 1 and not Experiments 2 or 3 (please, see Fig. S4 added to clarify our methodology and this point).

Reviewer: Due to this lack of clear framing, and particularly of partly inadequate methodology, I cannot recommend publication.

Response: We thank Reviewer#2 for this critical assessment. We recognise that in the previous submitted version the framing of CUE and the methodological description were not sufficiently clear. However, we have substantially improved the manuscript to address these points. These revisions strengthen the framing and clarify the Introduction and Methodology sections beside adding a schematic overview of the experimental design in supplementary file (Fig. S4, added to clarify our experimental design for each experiment). Thus, while we acknowledge the limitations of our study (we have detailed them in the Discussion section), we think that the revised manuscript provides robust and relevant insights into the resilience of Mediterranean soils under extreme heat stress and the effects of bioamendments (scientific literature is lacking or limited related to this topic). Moreover, please see our response to your previous comment.

Reviewer: I recommend reading Geyer et al. (2016) (DOI:10.1007/s10533-016-0191-y) to shed light on how incubation times impacts not only results, but also conceptualisation of CUE.

Response: Many thanks for the suggested reference. However, this study uses other methods for CUE calculation depending on the scale that is assessed. Nevertheless, although we recognize that these methods could be a valuable add to our study (as mentioned in the manuscript limitations section), we have used other method to quantify microbial CUE to evaluate the effects of the different bioamendments in two soils with contrasting properties. The method that we have used is widely used in the scientific literature as we mentioned in our previous responses (adding more references to support the use of this method to achieve our main objectives).

Detailed comments:

Reviewer: Can only Line 50-53; Line 6: Syntax errors

Response: We appreciate this comment, but we do not understand this comment to line 6:

“[α]These authors contributed equally to the manuscript and are considered co-first authors.”

Reviewer: L66-70: The formulations are a bit inaccurate here, and I have issues with the concepts. 1. “Consequently”, line 67; the death or dormancy, and the change in composition are responses of the community that partly define adaptation, not the cause of adaptation; death does not trigger a shift in metabolism, it IS a pretty dramatic shift in metabolism… high temperature is the cause of all that (death, dormancy, shift in metabolism and adaptation). 2. Also, “shift in metabolism to facilitate thermal adaptation”… I think a shift in metabolism is a form of adaptation (acclimation perhaps) itself, like species turnover and de novo genetic mutations

Response: We agree with Reviewer#2, this sentence could be confusing. We have reformulated this paragraph to be clearer following these comments:

“This often surpasses the microbial thermal optimum, resulting in the death or dormancy of thermosensitive taxa (Donhauser et al., 2020; Riah-Anglet et al., 2015) and changes in the community composition (Bérard et al., 2011; Hawkes and Keitt, 2015). These responses–together with physiological adjustments within surviving taxa–constitute microbial thermal adaptation and could be accompanied by altered metabolic activity, resulting in elevated respiration in the remaining thermotolerant species (Anjileli et al., 2021; Bardgett and Caruso, 2020). The alteration in soil microbial communities exposed to extreme heat stress often induces perturbation in organic matter mineralisation and C sequestration. Moreover, previous studies (Yang et al., 2023; Beugnon et al., 2025) have shown that heat stress reduces soil microbial C use efficiency (CUE; the proportion of C assimilated by soil microorganisms and allocated to biomass production rather than released as CO_2 through soil respiration), while simultaneously increasing respiration rates. This, in turn, can enhance organic matter mineralisation. In addition, soil C stocks depletion could dramatically occur under this situation if the soil does not receive sufficient C inputs. Microbial CUE plays a key role in soil C retention, soil organic C (SOC) storage and its global spatial variation, since it represents a dual microbial control point over both SOC accumulation (via biomass production) and SOC loss (via respiration; Tao et al., 2023). Therefore, microbial CUE is a more meaningful indicator of soil microbial functioning and C dynamics than respiration alone (Allison et al., 2010; Ghee et al., 2013; Li et al., 2019; Mganga et al., 2022)."

Reviewer: “inadvertently” is not the right word. increasing OM mineralisation, deleting soil C stocks and reducing CUE… it sounds like all those would be the direct consequence of an increased respiration in the remaining thermotolerant species. I think this is a large oversimplification. It needs to be laid out how increased mineralisation and decreased CUE may contribute to decrease C stocks, and in

which condition would this lead to a decrease in C stocks (with respect to plant C inputs particularly).

Response: We partially agree with this comment. First, we have deleted "inadvertently" (please, see our response to your previous comment). Then, our study is limited to the effect of heat stress in bare soil (without plants). We are focused on capturing the reaction of soil microbes to common heat stress in the areas of the study and the effects of bioamendments under these conditions (for soil fertility and microbial CUE). Nevertheless, we agree that the introduction of other factors as carbon input by plants could affect the carbon stocks in soil, but this is not the aim of the study. We have modified this paragraph to be more precise (please, see this modified paragraph in our previous response).

Reviewer: L70-74: how does the fact that CUE matters to C cycling and is sensitive to various factors justifies the need to understand resilience? We want to know specifically how resilience relates to soil C cycling, and how understanding CUE's response to drought and temperature is critical, because of this role in C cycling, to understand resilience…

Response: We appreciate this comment to enhance the clarity of the Introduction. We have introduced the meaning of Microbial CUE and how it collaborates in soil C cyclin under drought:

"…Moreover, previous studies (Yang et al., 2023; Beugnon et al., 2025) have shown that heat stress reduces soil microbial C use efficiency (CUE; the proportion of C assimilated by soil microorganisms and allocated to biomass production rather than released as CO_2 through soil respiration), while simultaneously increasing respiration rates. This, in turn, can enhance organic matter mineralisation. In addition, soil C stocks depletion could dramatically occur under this situation if the soil does not receive sufficient C inputs. Microbial CUE plays a key role in soil C retention, soil organic C (SOC) storage and its global spatial variation, since it represents a dual microbial control point over both SOC accumulation (via biomass production) and SOC loss (via respiration; Tao et al., 2023). Therefore, microbial CUE is a more meaningful indicator of soil microbial functioning and C dynamics than respiration alone (Allison et al., 2010; Ghee et al., 2013; Li et al., 2019; Mganga et al., 2022). Additionally, soil C sequestration and CO_2 released by soil microbes are directly affected by soil nutrients content according to Kirby et al. (2014). Moreover, other research support that microbial CUE strongly influences soil C sequestration and is sensitive to various biotic (e.g., competition between species) and abiotic (e.g., pH, temperature) factors (Iven et al., 2023; Tao et al., 2023; Jones et al., 2019) . However, there is a gap of knowledge related to soil management strategies

(bioamendments application) that may enhance the resilience of semi-arid soils to extreme heat-stress events (> 40 °C) to prevent widespread soil degradation (Ferreira et al., 2022)."

Also, we have modified another paragraph in the Introduction section to describe the importance of evaluating microbial CUE under extreme heat stress and drought conditions:

"Previous research has examined the impact of extreme heat waves on certain soils located in Mediterranean areas (Bañeras et al., 2022; Bérard et al., 2011). However, these studies do not assess the effects of extreme heat on these soils with challenging conditions for microbial CUE in arid or semi-arid regions, where periods of low moisture and aerobic conditions in soil are frequent, which tend to reduce microbial CUE (Zheng et al., 2019). Additionally, the presence of calcium carbonate in soils located in these regions limits microbial nutrient availability such as available phosphorus (P)."

Reviewer: L78: "challenging CUE conditions"… what are those? It was not mentioned before that calcareous soils have low CUE or why.

Response: we appreciate your comment to clarify the meaning of "Challenging CUE conditions", we have added further explanation: please, see our response to your previous comment. We referred to arid-semi arid regions (low soil moisture) and considerable carbonates content in soil that limits P availability and affects microbial CUE (so, fertilization is another key factor).

Furthermore, we have added more details about microbial CUE and calcareous soils. Please, find our modifications as follows:

"While P is essential for microbial growth, the application of inorganic P fertilisers could reduce microbial CUE in the short-term, as stated by Sánchez-Rodríguez et al. (2024). They found a significant decrease in microbial CUE (23–24%) in typical Mediterranean soils (Inceptisol, Alfisol, and Vertisol) when P was applied to the soil as diammonium phosphate or single superphosphate, which may be due to shifts in soil microbial community and nutrient dynamics or stoichiometric imbalances. However, Su et al. (2025) stated that the addition of organic fertilisers significantly enhanced CUE at the same time as increased P availability. These studies are fundamental for designing sustainable strategies that incorporate agricultural practices aligned with circular economy, such as compost application, to build more resilient farming systems (Moreno-Pérez, 2023) in accordance with European policies and strategies (Rato-Nunes et al., 2017). However, the effects on microbial CUE after the application of organic amendments under extreme heat stress is still not clear. "

Reviewer: L81: we need a ref to justify that low P availability would decrease CUE.

Response: thank you for your comment. However, this sentence was removed following Reviewer's 1 recommendation.

Reviewer: L82: compost application is a fairly common practice that is absolutely not unique to "organic agricultural practices".

Response: Many thanks for your comment. This paragraph has been reformulated to be more precise according to this comment:

"These studies are fundamental for designing sustainable strategies that incorporate agricultural practices aligned with circular economy, such as compost application, to build more resilient farming systems (Moreno-Pérez, 2023) in accordance with European policies and strategies (Rato-Nunes et al., 2017). However, the effects on microbial CUE after the application of organic amendments under extreme heat stress is still not clear."

Methods

Reviewer: L200-201: suddenly 14C is mentioned. I am not sure I understand here. The biosolids are obtained from commercial sources, so I guess they are not labelled with 14C. So how would one determine how much 14C from the biosolid has been incorporated into microbial biomass? Or is this using the natural abundance of 14C? but 14C as natural abundance is only useful to date centennial or millennial C, not the incorporation of new inputs into microbes which takes place over a few days to months…

Response: Many thanks for your comments. We acknowledge that the description of the methods may have been confusing. To clarify, the monitored ^{14}C in this study originated from the added ^{14}C-glucose, as stated in line 113 and described in Jones et al. (2018, 2019). The paragraph in question was intended as a disclaimer for readers, explaining the variation in the monitoring period of $^{14}CO_2$ release across temperatures (soil with bioamendments or mineral fertilizer). Since this is limited to both experiments 1 and 3, we initially placed it at the beginning of the methods. However, we have now moved this paragraph to the end of the methods section to improve the logical flow for readers.

"In our incubation experiments, microbial C uptake is defined as the total labelled C remaining in the system, which has not been respired as $^{14}CO_2$ or incorporated in the microbial biomass (Glanville et al., 2016; Sánchez-Rodríguez et al., 2024). Notably, since there is no universally accepted protocol for soil incubation experiments exploring extreme heat-stress in the presence or absence of bioamendments (Schroeder et al., 2021), the duration of the heat-stress events conducted in this study (to assess soil microbes response in Experiments 1 and 3) was designed to reflect the typical duration and intensity of heatwaves experienced in the region where the soil samples were collected, which can last over a week with daily air temperatures reaching up to 45.4 °C (see Fig.

S1). A mechanistic approach was utilised in our experiments, where the soils were maintained at high temperatures for one week to explore microbial responses under an extreme, worst-case scenario, in Experiments 1 and 3. In Experiments 1 and 3, the monitorization period (14CO2 measurements) was extended until $^{14}CO_2$ was not detectable in the NaOH traps (Experiment 1: 27-days under 20 °C and 30 °C and 21 days under 40 °C and 50 °C samples; Experiment 3: 16 days) because the timing of 14CO2 emissions stabilisation was strongly influenced by temperature, which explains the differences in duration between experiments and across the different temperatures. The $^{14}CO_2$ monitoring used technique captures $^{14}CO_2$ emissions from both catabolic (i.e., rapid mineralisation) and anabolic (i.e., slow $^{14}CO_2$ release due to cell turnover) processes."

Additionally, we have modified the last paragraph of the introduction section to make it clearer for readers according to this comment from Reviewer#2:

"*This study investigated the effects of a selection of bioamendments (composted olive mill pomace, composted biosolids, and composted solid urban residue) and a mineral fertiliser (diammonium phosphate) on microbial CUE and key soil chemical properties (including pH, labile C, N and available P) in two soils exhibiting contrasting carbonate and clay contents with a low P availability, a calcareous Vertisol and a non-calcareous Inceptisol, both collected from Mediterranean regions. We hypothesise that i) bioamendments will increase the availability of P and other nutrients supporting a more resilient soil microbial community with enhanced resistance to extreme heat-stress events than soils receiving mineral fertiliser or control soil with no P supply; ii) the enhancement of soil heat resistance will then increase microbial CUE in soils supplied with bioamendments; and iii) the calcareous Vertisol may exhibit a greater thermal and chemical buffering capacity under extreme heat events, supporting microbial metabolism at elevated temperatures more effectively than the non-calcareous Inceptisol, due to its higher pH and clay content, which help retain moisture. For that, three incubation experiments under controlled conditions were developed. Each experiment was designed to investigate different effects of extreme heat waves on soil microbes and functionality. In all cases, both soils were incubated at four different temperatures (20, 30, 40 and 50 °C). In the first experiment, soil respiration (measured as soil $^{14}CO_2$ emitted from soil following the addition of ^{14}C-labelled glucose) was monitored during and after a heat stress event and microbial CUE calculated for the different soil × treatment (control, mineral fertiliser and bioamendment) × temperature combinations, while the second experiment (same experimental design) was focused on the effects on soil chemical properties (without ^{14}C-labelled glucose application). A third experiment was conducted to assess the legacy effect of extreme heat-stress events on microbial activity in unamended soils, monitoring microbial activity after a heat stress when ^{14}C-labelled glucose was added."*

Reviewer: L202: what "monitoring period"? What "each experiment"? does this refer to each treatment (combination soil type/amendment)? Or each of the experiments numbered later?

Response: We appreciate your comments; we have clarified this section and moved this part just before statistical analysis according to your comments (once we have explained Experiment 1, 2 and 3). Please, see our modified paragraph in the previous comment.

Reviewer: L209: I am confused here: n=5, but further up (line 143): n=4. From the 4 times 100g prepared for each combination of bioamendment/soil type (40 pots in total: 2 soils x 5 bioamendments including no addition x 4 reps), line 143, how do we get to 5 replicates of bioamendment/soil type/temperature combinations?

Response: Many thanks for your comment. We understand that this section could be confusing. We have simplified and modified this section to be improved accordingly:

"Mixtures of the two soil types (Vertisol or Inceptisol) were prepared to explore the effect of mineral fertiliser vs bioamendments on microbial activity (Experiment 1) and soil nutrient cycling (Experiment 2) as a function of a simulated heat stress (20, 30, 40 and 50 °C). Consequently, the soils received varying quantities of mineral fertiliser or bioamendment, according to their P content (Table 2) to reach the target P level of 50 mg kg^{-1}, with a control treatment without mineral fertiliser or bioamendment. To ensure homogenisation after treatment (mineral fertiliser or bioamendment) application, larger mixtures of soil were prepared, because the experimental unit included only 2.5 g of soil. Therefore, soil was gradually added to pre-weighed fertiliser or bioamendment in larger batches, followed by thorough mixing to ensure homogeneity. Previously, the fertiliser and the bioamendments were ground and sieved to 0.5 mm to ensure homogeneity. From each homogenised batch, subsamples of each mixture (fertiliser / bioamendment and soil) were used for incubation experiments. In Experiment 3, soil (Vertisol or Inceptisol) was not mixed with any mineral fertiliser or bioamendment as we only evaluated the legacy effect of the heat stress (20, 30, 40 and 50 °C)."

Reviewer: L214: now I get it! 14C labelled glucose… so a glucose incorporation method is used as a standardised assay to quantify CUE.

Response: Exactly, in this work (as stated in the aims of the manuscripts and the methods) we used labelled ^{14}C glucose to monitor soil respiration in response to the heat stress in soils that were mixed (Experiment 1) or not (Experiment 3) with mineral fertilisers or bioamendments. This method is widely used to detect the shifts that could happens in microbial communities subjected to different external factors. The Introduction and Material and Material and Methods sections were deeply improved following Reviewer#2 comments and suggestions as can be seen in our previous responses and also here:

Introduction section: *"For that, three incubation experiments under controlled conditions were developed. Each experiment was designed to investigate different effects of extreme*

heat waves on soil microbes and functionality. In all cases, both soils were incubated at four different temperatures (20, 30, 40 and 50 °C). In the first experiment, soil respiration (measured as soil $^{14}CO_2$ emitted from soil following the addition of ^{14}C-labelled glucose) was monitored during and after a heat stress event and microbial CUE calculated for the different soil × treatment (control, mineral fertiliser and bioamendment) × temperature combinations, while the second experiment (same experimental design) was focused on the effects on soil chemical properties (without ^{14}C-labelled glucose application). A third experiment was conducted to assess the legacy effect of extreme heat-stress events on microbial activity in unamended soils, monitoring microbial activity after a heat stress when ^{14}C-labelled glucose was added."

In material and methods section:

Experiment 1: Microbial activity during and after an extreme heat-stress event

Microbial activity during and after an extreme heat-stress event was assessed by measuring microbial 14CO2 release and CUE, following the methods described in Jones et al. (2019, 2018) after the incorporation of 14C-labelled glucose in soils receiving inorganic fertiliser, bioamendments, or no addition (control; see Fig. S4 for a schematic overview of the experimental design)."

"Experiment 3: "To understand the legacy effect of the heat-stress, soil respiration was monitored after the heat stress (during 16 days) and microbial CUE calculated at the end of the monitoring period in another incubation experiment. Briefly, 2.5 g of soil (n = 5 per combination of soil type and temperature; control soil only without any mineral fertiliser or bioamendment) was placed in a sterile 50 ml polypropylene centrifuge tube, wetted with 200 µl of DI H2O, and pre-incubated for 1-week at 20 °C. After this week, soils were then placed in an incubator at 20 °C, 30 °C, 40 °C or 50 °C for another week. Then, soil samples were returned to 20 °C and 250 µl of 14C-labelled glucose (4.6 kBq ml-1, 10 mM; American Radiolabelled Chemicals Inc., St Louis, USA) was pipetted evenly onto the soil surface (see Fig. S4 Experiment 3 for an overview of the experimental design). NaOH traps were placed above the soil surface and changed on days 0.04, 0.13, 0.33, 1, 2, 3, 6, 8, 15 and 16, prior to measure 14CO2 via liquid scintillation counting. After 16 days, soil was extracted with fridge-cold 1 M NaCl to determine the amount of 14C remaining in the soil and microbial CUE calculated as described previously in Experiment 1."

Additionally, we have added a new figure (Fig. S4) including detailed information of each incubation experiment, summarising the experimental design in each case (Experiment 1, 2 and 3). We hope that this figure helps the readers understand our study and improve our manuscript.

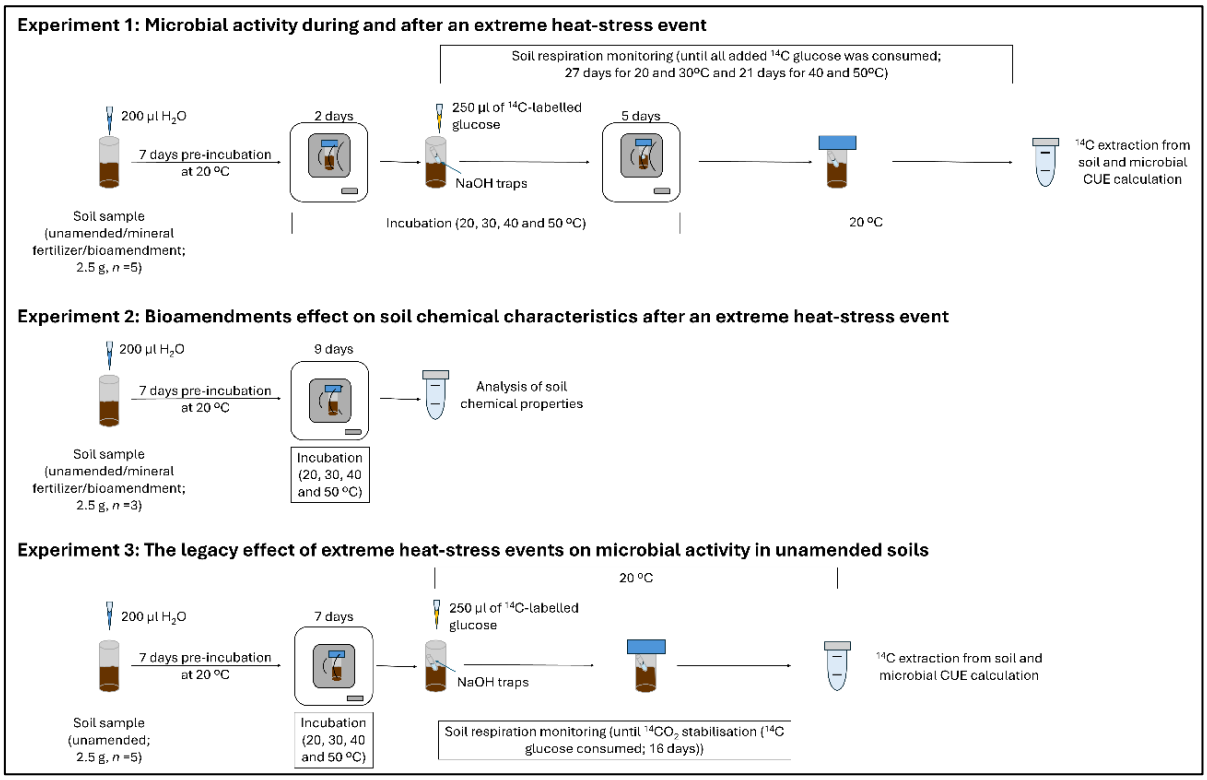

Fig. S4. Schematic overview of the experimental design. Experiment 1: Microbial activity during and after the heat stress (20, 30, 40 and 50 °C) was applied to the soils with the different treatments (control, inorganic fertiliser, or bioamendments). Experiment 2: Soil chemical properties (soil pH, EC, moisture, total N and C, ammonium (NH_4^+), nitrate (NO_3^-), and Olsen-P) as a function of the soil and treatment (control, inorganic fertiliser, or bioamendments) after the heat stress (20, 30, 40 and 50 °C). Experiment 3: The legacy effect of heat stress was evaluated in unamended soils after the heat stress (20, 30, 40 and 50 °C), followed by ^{14}C-glucose addition.

Reviewer: L225: so when is incorporation into microbial biomass quantified? I suppose the extraction with NaCl extracts what is not in microbial biomass or respired? So one would need to deduce that from what is added originally and what is recovered as 14CO2 cumulatively to deduce what is in microbial biomass?

Response: We appreciate this comment. The method that we use in our study to calculate microbial CUE, following Jones et al. (2019, 2018) and Sánchez-Rodríguez et al. (2024), estimates microbial immobilisation by using the following equation:

"Additionally, microbial immobilisation of the ^{14}C-substrate ($^{14}C_{imm}$) after the monitoring period was estimated as follows:

$$^{14}C_{imm} = {}^{14}C_{tot} - {}^{14}C_{NaCl} - {}^{14}CO_{0-t\ days} \qquad (1)$$

As Reviewer#2 mentioned, the extraction with 1 M NaCl is used to quantify the amount of ^{14}C recovered from the soil at the end of the experiments.

Reviewer: L239: In experiment 1, 7 days at the different temperatures, including only 5 in the presence of 14C glucose. Ok; but why 9 days in experiment 2?

Response: As Reviewer#2 commented, the incubation time in Experiment 1 and 3 is different from that used in Experiment 2. For clarification, all the samples of the three-experiment passed through 7 days of preincubation at 20 ºC and then, they were incubated at different temperatures.

It is important to realise that the three experiments have different objectives (see Material and Methods, in the description of each experiment, separately). Experiment 1 and 3 aimed to monitor the changes in soil respiration during and after the heat stress in both soils (Vertisol and Inceptisol) unamended (Experiment 3) and amended with the mineral fertiliser or bioamendments (Experiment 1), and after the heat stress in Experiment 3

Experiment 2 was focused on evaluating modifications in soil chemical properties after the heat stress. For that, the duration of the heat stress do not have to be exactly the same as in Experiment 1, and we decided to increase this time in two days (so, the heat wave in this case was simulated for 9 days; still within the values observed in our climatological data).

In conclusion, the methods were adjusted depending on the planned objectives in each experiment.

Please check our new version of the Introduction (last paragraph) and Material and methods where these changes were done according to this and other comments (see our responses to other suggestions to avoid repetition here) – this information was added in a previous response, so, to avoid repetition we have not included it again here.

Reviewer: L254: and now it's a week of heating. But how long are soils at 20oC before 14C glucose addition?

Response: Many thanks for your comment. We think this section is clearer now thanks to that. Please see our schematic overview of the experimental design, now included as a new figure in supplementary material (Fig. S4).

Summarizing, the samples (Experiments 1, 2 and 3) were wetted and preincubated for 1 week at 20 ºC before the incubation at different temperatures. Experiments 1 and 3 used labelled ^{14}C glucose but it was not used in Experiment 2. In Experiment 1, labelled glucose was used with the main aim to detect the effect of heat stress during and after heat stress in soils previously amended with the different treatments; the samples were incubated for two days with different temperatures before the addition of ^{14}C-labelled glucose and after that the incubation was prolonged to 7 days. In Experiment 2, the samples (mixed with bioamendments or mineral fertiliser) were heated for 9 days to reproduce extreme field conditions (like those observed under real conditions) without any supply of ^{14}C-labelled glucose, as the objective was to evaluate alterations in soil chemical properties. Then, in Experiment 3, we used labelled glucose with the aim to detect the legacy effect of heat stress on unamended soils (after the heat stress); the soil samples were incubated at different temperatures for 7 days to be returned to 20ºC and

then ^{14}C-labelled glucose was added to measure microbes' respiration and calculate microbial CUE (at the end of the experiments).

Reviewer: L256-257: as I suspected line 224-225, microbial biomass C of microbial biomass 14C were never measured, only the remaining 14C in the total soil is quantified. Explanations about how this is used to calculate CUE are needed! It says here "As described above" but I can't find the calculations/equations anywhere.

Response: We appreciate this comment. As we previously mentioned, there are various methods that are used to estimate the amount of C that remains in the soil, however, in this research we have used the method described by Jones et al. (2019, 2018) to quantify the CUE, using the formulas:

"Additionally, microbial immobilisation of the ^{14}C-substrate ($^{14}C_{imm}$) after the monitoring period was estimated as follows:

$$^{14}C_{imm} = {}^{14}C_{tot} - {}^{14}C_{NaCl} - {}^{14}CO_{0-t\ days} \qquad (1)$$

where $^{14}C_{tot}$ is the total amount of ^{14}C-substrate added to the soil, $^{14}C_{NaCl}$ is the amount of ^{14}C recovered from the soil in the 1 M NaCl extracts at the end of the experiments and $^{14}CO_{0-t\ days}$ is the total amount of ^{14}C recovered as $^{14}CO_2$ during the experiments (21-27 days). Then, microbial CUE for the ^{14}C substrate was estimated as follows following Jones et al. (2018a,b):

$$microbial\ CUE = {}^{14}C_{imm} / ({}^{14}C_{imm} + {}^{14}CO_{0-t\ days}) \qquad (2)"$$

Reviewer: L258-260: Now I am a bit confused. L256, it is implied that all treatments are incubated for 16 days before extraction for remaining 14C. For experiment 1, lines 217-220, it is indeed indicated that incubation time difference between temperature treatments. So this seems to apply only to experiment 1. I think diverging incubation time for calculating CUE based on glucose incorporation are hugely problematic, as described in the general comment, and I question the validity of the approach to conclude anything g about the effect of temperature on CUE.

Response: Many thanks for your comment. As described for each experiment (and included in previous responses), the monitoring of $^{14}CO_2$ was stopped once the rate of $^{14}CO_2$ had plateaued indicating the all the glucose added was mineralised. At that point, microbial CUE was evaluated. This approach allows us to capture the effect of the heat stress on microbial CUE before microbial adaptation and to minimize CUE overestimation. Since each temperature has a different impact on microbial activity—and higher temperatures accelerate glucose consumption and C sequestration—it seems more logical to adapt the monitoring CO_2 period to these slight differences (just a few days) for the different temperatures and calculate microbial CUE immediately at this point. This method is commonly used in similar studies to evaluate the impact of different treatments

on soil C, respiration and CUE (please, see the references that we have added in our previous responses dealing with this issue).