

Interactive comment on “An unknown respiration pathway substantially contributes to soil CO₂ emissions” by V. Maire et al.

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The ms consists on the results of 3 studies evaluating importance of exoenzymes to CO₂ release from soil. The individual studies are probably well done, despite they are based on numerous unproven assumptions. Also the enzymes were carefully chosen for the experiments. My main problem is that the study pretends to show new pathways of organic C decomposition in soil and new processes contributing to soil CO₂ emissions. To my knowledge, it is well known that exoenzymes contribute to the decomposition, hydrolysis, and oxidation of organic substances outside of microbial cells. This is not new and should be not tried to sell as a new pathway of C cycle. However, I appreciate the authors that they devoted to this problem, because despite the general

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knowledge, there are only very few studies focused on the separation / evaluation of processes occurring outside and inside soil microorganisms.

Authors: After reading this general comment we understood that our ideas were not sufficiently introduced. However, we maintain as a novelty the fact that complex biochemical reactions underpinning respiration can occur in the soil without compartmented living structure. Comments from other referees do not leave any doubts on this question. We agree that it is well known that exoenzymes contribute to the decomposition and hydrolysis of soil organic matters for their assimilation by micro-organisms. However, we are not aware of any metabolic process conducted by exoenzymes that lead to the oxidation and particularly the emission of CO₂ of soil organic matter. To our knowledge, only the endoenzymes are able to conduct such oxidative metabolic process. It is widely accepted by the scientific community that these endoenzymes could not persist outside cells. To support this assertion we can refer, for example, to Burns who wrote in SBB in 1982 about these endoenzymes that he classified in Category 1 'these enzymes cannot function outside the cell because they depend on various co-factors, on being located adjacent to other enzymes, or on some physiological property of the cell (e.g. redox potential)'. The introduction has been completely rewritten in order to precise on which part of the decomposition process we focus on this study (respiration) and on which enzymes (endoenzymes).

Therefore, it is much better to show to which portion this (well known) CO₂ source contributes to the CO₂ fluxes from various soils and how this contribution depends on the soil properties (if this is the case). For this evaluation of the contribution however, the underlying assumptions for the methods/experiments should be proven and clearly presented. Furthermore, the main part of the Results is written too concise. It is hard to evaluate the details. The ms should be completely rewritten, including enlargement of the Results section, separate Discussion based on the results obtained, and should not overestimate the things that are already known.

General comments Using of terms: there are some terms that the authors use incor-

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rectly. Such terms as 'metabolism', 'respiration' . . . are the terms for solely intracellular processes. It is not correct to expand these terms on extracellular processes, even they are biochemically/enzymatically driven. E.g. 'respiration' is not equal to 'CO₂ production'. The Introduction is very weak: it contains just 20 lines, and consists mainly on general words. There is no information, WHY and WHICH enzyme groups (and not only hydrolytic enzymes) are released extracellularly.

Authors: We agree that introduction deserves a better explanation on the different kinds of enzymes implied in the organic C decomposition, their role and if they are specifically endo or exoenzymes. As suggested, we have now completed the introduction in this way.

The Title should reflect the study. It should be clear from the Title that contribution of extracellular enzymes to CO₂ production in soils were evaluated.

Authors: As explained above our study demonstrates the existence of an unknown oxidative metabolism. We agree that the term "respiration" was not entirely adapted because typically reserved for living organisms. We therefore propose the following title "An unknown oxidative metabolism substantially contributes to soil CO₂ emissions".

Section 2.3.3: the approach of separate evaluation of the activity of soluble and soil-immobilized enzymes is not clear. For soluble enzymes the soil was incubated 5 min and centrifuged thereafter. For the immobilized enzymes the soil was incubated between 5 and 45 min and centrifuged thereafter. Is the duration of the incubation the only difference? If yes, this is surely not enough to separate the activity of immobilized enzymes. This part needs extended explanation with all details and assumptions to evaluate the plausibility. This is very important as a part of the main hypothesis should be proven by the results of this approach. Fig 2. The approach is not clear for separation of the activity of total and immobilized enzymes. If I understood correctly, the activity of immobilized enzymes was calculated by difference. Consequently, all experimental errors are included in the activity of immobilized enzymes.

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Authors: We agree that the approach developed to separate activity of soluble and soil-immobilized enzymes was not sufficiently explained. Of course, the duration of the incubation is not the only difference between the two methods. Activity of soluble enzyme (SolEnz) was quantified after their extraction from soil. For extraction, 80 mg soil samples were mixed with the 300 μ l of the buffer solution containing substrates, co-factors and intermediate enzymes (See section 2.3b) and shaken during 5 mn. Samples were then centrifuged at 11,000 x g during 3 min. The supernatant containing soluble enzymes, co-factors and substrates was transferred into a micro-plate where activity of soluble enzyme activity was measured during 3 min. The production rate of NADPH (for GHK and G6PI) and the consumption rate of NADH (for MDH) consecutive to the activity of soluble enzymes were quantified by spectrometry at 340 nm. For measurement of total enzyme activity (TotalEnz), the enzymatic reaction was made into the soil, that is, in presence of soluble and soil-immobilized enzymes. To this end, soil-enzyme mixture was incubated with substrates, cofactors (See section 2.3b) during 45 min. At different times between 5 and 45 min of incubation with substrates, independent samples were harvested and centrifuged at 11,000 x g during 3min. The NADPH concentration in the supernatant was determined by spectrophotometry at 340 nm. The production of NADPH (for GHK and G6PI) or the consumption of NADH (for MDH) during the 45 min incubation of soil with substrates corresponded to the activity of total enzymes. Finally, activity of soil-immobilized enzymes was estimated by difference ($\text{ImmEnz} = \text{TotalEnz} - \text{SoluEnz}$). This section has been completely rewritten in the new version of the manuscript (P9-10). We confirm that activity of soil-immobilized enzymes was estimated by difference ($\text{TotalEnz} - \text{SoluEnz}$) signifying that all experimental errors are included in this activity. However, activity of soluble enzymes is close to zero after 15 days of incubation. Therefore, we are confident in our estimation of fraction of enzymes that are stabilized on soil particles. Acceptance of CO₂ release from g-irradiated soil as Exomet is very questionable (8671/ 1. . .). G-irradiation surely kills the microorganisms. This leads to the outflow of endoenzymes into the soil. These artificially released enzymes surely may contribute to oxidation of organics and

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consequently CO₂ release. However, may be important part of these enzymes were not present in the soil before g-irradiation? (Sorry, I just have seen that this issue is already mentioned by the authors in the next lines).

One topic regarding the enzyme stability is completely disregarded. As enzymes are easily available proteins, after their release into the soil, the most part of them will be decomposed and utilized as substrates by living microorganisms. So, in many cases when non sterilized soil was used, as well as under real soil conditions, the released enzymes will be not stabilized, but decomposed by other enzymes and microorganisms. This surely has effects on their activity and contribution to CO₂. 8671/ 21 This is also big question. It is not really clear, does g-irradiation affect the exoenzymes or not? 8672/ 7 The exoenzymes may diffuse, but this process is really of very low importance, as they will be bound on clay minerals or SOM very fast.

Authors: These questions were already largely discussed in the previous version of the manuscript. These informations are available P12 of the new manuscript and below.

By fixing the same Rx in the irradiated and non-irradiated soils, the model assumes that irradiation has no effect on the pre-existent EXOMET Rx. We discuss here two examples where irradiation could modify Rx. First, the γ -irradiation by denaturing part of soil enzymes could decrease Rx. It is easy to show in this case that the EXOMET contribution to soil CO₂ emissions is underestimated by the model. Nevertheless, this underestimation of EXOMET is likely to be moderate since the effect of γ -irradiation on soil enzymes is typically low (see review of McNamara et al., 2003). Second, the irradiation by suppressing the microbial uptake of organic substrates could increase the availability of these substrates for EXOMET increasing Rx. In this case, the current model would overestimate the EXOMET contribution to soil CO₂ emissions. However, EXOMET and living respiration are not likely to be in competition for organic substrates. Indeed, EXOMET may have preferential access to organic substrate since EXOMET-carrying enzymes are adsorbed on soil particles including organic matter. Moreover, most of the soil sites where EXOMET can proceed are deprived of microorganisms.

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Indeed, enzymes responsible for EXOMET may diffuse in most soil pores whereas living soil microorganisms due to their size occupy less than 0.5% of the soil pore space (Paul and Clark, 1989).

Specific remarks 8664/ 5 and 8665/ 10 this is very uncertain question: The microorganisms usually do not die. They can be grazed by soil animals (e.g. amoebae), but in this situation the most part of the microorganisms (including enzymes) will be digested. If the environmental conditions change or the substrate is exhausted, the microorganisms convert to the dormant state and build cysts or spores. So, one of the assumption of the study, that exoenzymes are released into the soil just 'by case' by dying probably is not very consistent. 8678/ 15 Again, the microorganisms actually not die from alone. Therefore, this delay is not really clear compared to what.

Authors: We strongly disagree with this assertion that microbial cells never die or are never lyzed. There are many factors causing microbial death and cell lysis. Viral infection, freeze-thaw and wet-dry cycles, toxic compounds secreted by plants are some examples (Henry, 2007; Weinbauer, 2004; Wommack KE, Colwell 2000 ; Suttle 2005). The viral lysis of microbial cells could even affect between 10 and 40% of cells. Finally, to support this idea that cell lysis is a common process, we propose to observe fig below which represents number of damaged (2 on Fig) and undamaged cells (1 on Fig) in the non-irradiated soil from Theix. Half of cells are damaged and probably released their cytoplasm.

8664/ 17 this is generally not correct: it is well known that exoenzymes strongly contribute to the decomposition of organics, and this is the main pathway of polymer decomposition.

Authors: This part of manuscript has been completely rewritten.

8664/ 19 this is the chemical / thermal decomposition. In soils however, the glucose will be take up in microbial cells and will be decomposed by enzymes.

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Authors: same comment.

8664/ 25 the respiration is surely intracellular process. However, there are various pathways of C oxidation and consequently of CO₂ production independently on the intracellular oxidation. Extracellular decomposition of polymers is one of them. 8665/ 5 this is not really correct: besides hydrolytic enzymes also oxidising enzymes (oxidases, peroxidases) are present in soils extracellularly and these enzymes contribute to CO₂ production.

Authors: We disagree with this assertion. As explained in our first response, we are not aware of any extracellular decomposition of polymers that conducts to CO₂ production. It should be very helpful if the reviewer quotes any study that demonstrates such a pathway.

8665/ 1 strong reduction does not mean that the respiration will be completely stopped. 8665/ 7 again: minimization does not mean absence

Authors: We agree that a small pool of soil microorganisms may have been undetected in these studies. This is the reason why we developed intensive research to detect presence of active microorganisms after irradiation and to quantify their possible contribution to CO₂ emissions from irradiated soils. Our investigations presented in the appendix clearly demonstrated that emissions of CO₂ from irradiated soil could not be explained by undetectable gamma-resistant microbes, unless considering unrealistic cell respiratory activity. We modified the writing of our introduction in order to discuss about the possible contribution of gamma-resistant microorganisms to CO₂ emissions and to better introduce our assertion that "To date, the cause of CO₂ production in soils where microbial life has been minimized is unknown and questions our basic knowledge of biology"

8665/ 26 because the soil from individual samplings were bulked together, all further analyses are expressed with analytical error/variation, and not the real variation in the field. Figs 2,3,4 It is not clear what the error bars present. In any case, they show the

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variability of analytics, not of the real biological variability of the parameters, as the soil samples were mixed.

Authors: The variation of EXOMET at field scale is not the topic of this study. Here we identify a new respiration pathway and quantify its potential contribution to soil CO₂ emissions from different soil. Error bars presented in this study include analytical AND experimental variability. Our method of soil sampling and soil pre-treatment before its use for experiment are (was) clearly explained P5 of the manuscript.

8667/ 13 what is UMDH?

Authors: U is abbreviation of the international standard "unit" for enzymatic activities and MDH the abbreviation of malate dehydrogenase. These abbreviations were defined later in the text owing to a previous restructuring of the text. This error has been corrected (P6 L130-136).

8667/ 21-23 the amount of glucose added to the soil is very high!

Authors: This amount of glucose has been set up to place the microcosm under unlimiting condition of substrate for respiratory enzymes supplied with the yeast extract.

8667/ 28 how CO₂, O₂ and ¹³CO₂ were measured? Continuously, trapping, sampling at some periods?

Authors: The details on flux measurements were specified in section 2.6 of the Method. We have now referred to this section L146.

8670/ 21 This equation consists on 6 (!) parameters. Using 6 parameters it is easy to fit any data points. Additionally, the parameters of such equation are interdependent. So, the comparison of these parameters is hardly possible unless half of the parameters will be estimated by independent approach. Table 2 is superficial, as the parameters of the non-linear regressions are highly interdependent, and it is not correct to calculate 6 parameters if only 8 experimental points are present. Fig 2 An approximation of 8 points by equation with 6 parameters is incorrect (and the parameters are highly

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interdependent). For each parameter at least 2 points should be available. 8676/ 6 I am not sure that this is the half life of the fast pool. In my view it is the half life of enzymes denaturation after there are released outside of the cells. Authors: We agree that presentation of our model and its use to quantify decay rate and size of enzyme pools deserved more attention and explanation. However, I would like to confirm here that the inclusion of three pools of enzymes is essential to model kinetics of enzymatic activities. As explained in our manuscript a substantial part of the initial enzymatic activity was lost within minutes following enzyme addition to the soil (the difference between initial activity and that at the first measurement 20 minutes after enzyme addition, Fig. 2). These enzymes are likely denatured by physico-chemical processes (Burns, 1982). Decay rate of the fast pool was so rapid it could not be characterized precisely in this experiment. Consequently, size a and decay rate b of the fast pool were fixed to the amount of enzymatic activity lost during the first 20 minutes of incubation and to $1/5 \text{ min}^{-1}$. Then, parameters c , d , f and g of intermediate and slow pools of enzymes could be estimated using classical non-linear regression procedure. We verified that uncertainty on decay rate of fast pool has negligible effect on estimation of parameters of intermediate and slow pools. This lack of effect on estimation of parameters is explained by the strong difference between decay rate of fast pool and those of intermediate and slow pools of enzymes (Data not shown).

8671/ 18 This model equation is not correct. It should be $R_{ni} = \dots$ (not R_i)! Please check and correct!

Authors: Apologies! Equation should be read as follows: This point has now been fixed.

8673/ 1 why it was necessary to sample the microcosms to estimate CO₂ emission? The CO₂ can be measured by GC or by trapping without microcosms' sampling.

Authors: Destructive sampling was preferred to gas sampling to maintain sterility of microcosms. Moreover, sampled microcosms were used to verify maintenance of sterility

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throughout the incubation period. This is specified P13 L295-296.

8675/ 1 these high numbers are mainly the result of very high glucose amount added to the soil.

Authors: The significant point is the presence of activity rather than the magnitude of response, which we agree could be somewhat lower with lower glucose inputs. However, the addition of high glucose gives an indication of the potential response in soil. Moreover, our results show that the persistence of this EXOMET depends on dynamics of endoenzymes which are continuously inactivated except a small fraction that is stabilized in soil (P16 L367-374).

8675/ 9-20 this paragraph is poor speculation. If this process sequence occurs within the cells, the individual steps are finely adjusted to each other spatially and timely. Outside of the cells it is hardly to believe that such or even comparable correspondence is possible in chaotic distribution of organelles after the cell died (if they died).

Authors: This paragraph is absolutely not speculation but a summary of our results. I propose you to reanalyse each sentence and to link it with results:

1/Our results confirm the idea that the enzymatic cascade leading to the oxidation of glucose (i.e. glycolysis and the Krebs cycle) can occur in an extracellular context in soil and water.

I will not recall here all methods and controls made to ensure sterility of microcosms, all this information are available in our appendix.

Has the glucose been decomposed in our controls (irradiated soil + glucose, sterilized water + glucose)? Fig 3 clearly indicates NO.

Has the supply of cell-free yeast extract containing respiratory enzymes triggered enormous respirations fluxes (O₂ consumption, CO₂ consumption) in water and soil microcosms? Fig 3 clearly indicates NO.

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We conclude that the enzymatic cascade leading to the oxidation of glucose can occur without any cellular organization. Is it false?

It is interesting to note that referee 3 has himself observed production of CO₂ from a cell-free yeast extract though he has never published it.

2/ This indicates that respiratory enzymes can maintain their activity outside the cell and have access to substrate and co-factor flux to function.

This sentence is only consequence of what preceding. Are endoenzymes able to function without co-factors and substrates? Of course not.

3/ It is worth noting that the electron transfer to O₂ was maintained since the emission of CO₂ was coupled to the consumption of O₂ (Fig. 3A).

There is no doubt that the transformation of O₂ in CO₂ requires a transfer of electrons.

4/ This electron transfer, probably carried out by the cytochromes of cellular debris (Trevors et al., 1982), permit the regeneration of electron acceptors (i.e. NAD⁺) explaining the persistence of EXOMET over 53 days (Fig. 3).

Here we speculate how the electron transfer is carried out in the absence of cells. However, by using the conditional verb form we clearly inform the reader that this is our interpretation.

5/ Moreover, our results show that fermentative metabolism is another intracellular process that can be reconstituted outside the cell when the availability of O₂ for the Exomet is low.

Is fermentative metabolism another intracellular process that can be reconstituted outside the cell?

The increase in flask pressure (P15 L355-356) and the unbalance between O₂ consumption and CO₂ production (Fig 3) at the beginning of yeast-extract incubation in water with glucose provide univocal response on this question.

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8676/ 14 The Fig shows only the decrease of the enzyme activity. It does not show the immobilization of enzymes.

Authors: Fig 2 not only shows the decrease of the enzyme activity but also separate activity of soluble and soil-immobilized enzymes. Figure 2 clearly shows that the long-term persistence of enzymatic activities exclusively relied on enzymes immobilized on soil particles. Again we present only experimental facts.

8676/ 23 was the sterility proven at the end of incubation? 8677/ 10 was the sterility proven at the end of incubation?

Authors: The sterility has been checked at each date of the experiment (see the appendix).

8677/ 17 CHCl₃ does not completely kill microorganisms in soil. A part of microorganisms remains alive (e.g. Kemmitt et al. 2008)

Authors: Please read correctly our experimental plan (P13 L302 – P14 L315, caption of fig 6). Chloroform fumigation was applied in combination to irradiation. Irradiation was used to sterilize soils and allow measurement of EXOMET. Then different treatments (fumigation etc) were applied to this irradiated soil in order to determine their effect on EXOMET.

8678/ 22 The origin of life in soils, means in initial soils at the coasts and tidal locations, is generally accepted. However, the conclusion presented here (8678/ 28) is not correct, as the study was focused on the enzymes released by microorganisms (consequently microorganisms / life originated earlier than the enzymes released) and not on the origin of enzymes without microorganisms. May be it sounds as nice conclusion for the paper, but the study and the results are not connected with it.

Authors: All theories developed to explain the origin of life have their part of speculations. When Miller and Urey demonstrated in 1959 that it is possible to produce a wide variety of organic molecule in conditions thought to be similar to those of the early

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earth, they open some doors to understand how life could have appeared. Nevertheless, the chemical structure of their amino acids etc is ridiculously simple compared to that of living organisms and I am sure that skeptics of this period underlined it. In this article, we try to modestly contribute to the issue of self-organization of life building block leading to first ancestral metabolism or life form. Previous studies have shown the role of soil minerals in the concentration and polymerization of amino-acids and sugars leading to the formation of a wide variety of molecules (proteins, polysaccharides) during the prebiotic period (Bernal, 1949; Miller and Urey, 1959). However, these models do not explain how you pass from macro-molecules to metabolism. Our results indubitably provide a part of response to this question with one example: the oxidative metabolism. Indeed, we show that complex biochemical reactions underpinning bioenergetics of life (respiration) can occur spontaneously in soil without compartmented living structure. Of course, all relevant organic molecules (comprising proteins with a catalytic function) must be present in soil, but this is the speculation part of our theory. Finally, we do not say glycolysis and the Krebs cycle have appeared in soil before the presence of the first cells. We know that these metabolisms have been optimized with evolution. However, on the basis of our observations, we say that the first ancestral oxidative metabolism may have occurred in soil before the presence of the first cells. This paragraph has been slightly modified in order to clarify our modest contribution to this big question for humanity.

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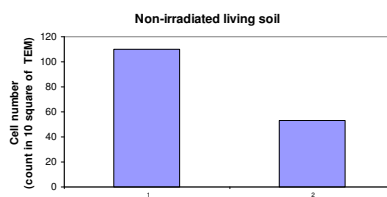


Fig. 1.

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