



RESEARCH NOTE

**REVISED** Unexpected results in Chernozem soil respiration while measuring the effect of a bio-fertilizer on soil microbial activity [version 2; referees: 2 approved]

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**Abstract**

The number of studies investigating the effect of bio-fertilizers is increasing because of their importance in sustainable agriculture and environmental quality. In our experiments, we measured the effect of different fertilizers on soil respiration. In the present study, we were looking for the cause of unexpected changes in CO<sub>2</sub> values while examining Chernozem soil samples. We concluded that CO<sub>2</sub> oxidizing microbes or methanotrophs may be present in the soil that periodically consume CO<sub>2</sub>. This is unusual for a sample taken from the upper layer of well-ventilated Chernozem soil with optimal moisture content.

**Open Peer Review**

Referee Status:

|   | Invited Referees |                |
|---|------------------|----------------|
|   | 1                | 2              |
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**Author roles:** **Bautista G:** Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Mátyás B:** Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Carpio I:** Methodology, Project Administration, Writing – Review & Editing; **Vilches R:** Investigation, Methodology, Writing – Review & Editing; **Pazmino K:** Investigation, Methodology, Project Administration, Writing – Review & Editing

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**REVISED Amendments from Version 1**

We extended the Introduction chapter to clarify the importance of measuring physical and chemical soil properties when examining soil microbiological activities.

We extended the Methods chapter and named the methods and required sample preparations that were applied for measuring the main soil properties.

Dataset 1 was replaced with Table 1 in the main text. This was also updated with a new parameter, Total Nitrogen, according to Prof. Muhammad Aslam Ali's advice.

We responded by comments to Prof. Muhammad Aslam Ali's questions related to the experimental setups, and marks of the figures.

We named 3 more co-authors who strongly contributed to the measurements of the physical and chemical soil properties.

**See referee reports**

## Introduction

The soil can be characterized by physical, chemical and microbiological properties<sup>1-4</sup>. The quantitative (microbial biomass, number of bacteria)<sup>5,6</sup> and qualitative (enzymatic activity, soil respiration)<sup>7,8</sup> microbiological properties of the soil greatly contribute to the impact analysis of land use<sup>9-11</sup>, nutrition<sup>12</sup> and soil management<sup>13</sup>. Research related to the benefits of microbes as biofertilizer has become increasingly important in the agricultural sector. This is due to the possibility of achieving higher crop yields while minimizing negative impact on the environment. It is well known that bio-fertilizers increase plant yield and improve soil fertility<sup>14-16</sup>. Soil respiration is an important indicator of soil microbial activity<sup>17,18</sup>. In our experiments, we measured the effect of different chemicals<sup>19-22</sup> and a bio-fertilizer on soil microbial activity, using both well-established and novel methods under laboratory conditions. We present some unexpected results from a setup in which Chernozem soil samples were examined.

## Methods

### Sampling site

A total of 24 soil samples were collected near Debrecen, Hungary, on the 19th April 2016, from an upper layer (0–20 cm) of Chernozem soil (47° 33' 55.36" N; 21° 28' 12.27" E).

### Treatment

The phylazonit bio-fertilizer (produced by Phylazonit.Ltd, Hungary) with the following composition: *Bacillus megaterium*, *Bacillus circulans*, *Pseudomonas putida*, was tested (15 l/ha) in an optimized ratio for soil injection. Number of bacteria: 10<sup>9</sup> piece/cm<sup>3</sup>.

### Soil properties

Soil moisture content was determined gravimetrically, drying the soil at 105°C for 24 hours according to Klimes-Szmik's method (1970)<sup>23</sup>. Silt and clay fractions were measured by the settling

method<sup>24</sup>. We measured the Arany-type plasticity index according to Stefanovits (1975)<sup>25-27</sup>, while the minimal water capacity and soil texture were determined by Klimes-Szmik's method<sup>23</sup>. To measure the chemical properties of the soil, the samples were sieved through 2mm mesh and pre-incubated at 25°C for 72 hours. Soil pH in distilled water and in 1M potassium chloride KCl (soil/water, 1/2.5, w/w) were determined according to Buzás (1988)<sup>24</sup>. The electrical conductivity (EC) (soil/water, 1/5, w/w) was then determined with a glass electrode according to Kong *et al.*, 2013<sup>28</sup>. The hydrolytic acidity (y1) was measured according to Buzás (1988)<sup>24</sup>, while the concentration of NO<sub>3</sub><sup>-</sup>-N was determined according to Felföldy (1987)<sup>29</sup>. Total nitrogen was determined according to Kong *et al.* (2013)<sup>28</sup>. Nitrate exploration was carried out after 14 days incubation according to Felföldy (1987)<sup>29</sup>. We determined AL-P<sub>2</sub>O<sub>5</sub> and ALK<sub>2</sub>O based on Szegi's method (1979)<sup>30</sup>. The humus content was determined using potassium dichromate according to Székely (1988)<sup>31</sup>. Total number of bacteria was counted in bouillon agar using the plate dilution method (Szegi, 1979)<sup>30</sup>. We measured the organic carbon concentration in K<sub>2</sub>SO<sub>4</sub> extract, following the protocol in Székely *et al.* (1988)<sup>31</sup>. Microbial biomass carbon (MBC) was measured using the chloroform fumigation-extraction method. Soil samples were fumigated by adding alcohol-free chloroform at 25°C for 24 hours. The fumigated and unfumigated soil samples were extracted with 50 ml 0.5 M potassium sulfate (K<sub>2</sub>SO<sub>4</sub>) according to Vance *et al.* (1987)<sup>32</sup>. The following formula was applied to calculate the MBC (Kong *et al.*, 2013)<sup>28</sup>:

$$\text{MBC} = 2.22 \times \text{EC}$$

where EC = organic C extracted from fumigated soils – organic C extracted from unfumigated soils (Table 1).

### Soil respiration

The experimental design was completely randomized, treatments were incubations (25°C). An OxiTop OC110 respirometer was used to quantify the release and capture of CO<sub>2</sub> that is automatically determined by the device after the biological oxygen demand (BOD) required for the degradation of organic matter has been measured. We used a 500 ml glass bottle system following the instruction manual (<https://www.wtw.com/en/service/downloads/operating-manuals.html>). 10g of soil sample were placed into OxiTop flasks, and capped with the sensor heads according to Barrales-Brito *et al.* (2014)<sup>33</sup>. 2.5g of CO<sub>2</sub> absorber (sodalime) were then added to a tank to absorb the generated CO<sub>2</sub><sup>33</sup>. An induced method was also used, in which 0.1g glucose was added to the soil samples. Each treatment was replicated four times. As Figure 1 shows, four samples were always measured in parallel: Absolute control (does not contain fertilizer, nor added glucose), Induced control (contains added glucose), Treated (contains bio-fertilizer) and Induced treated (contains bio-fertilizer and glucose). The Oxitop automatically provides the values related to CO<sub>2</sub> production according to the pressure change measured by its sensor (there is no need to carry out titrations or any additional work).

**Table 1. Average values for a number of different soil properties.**

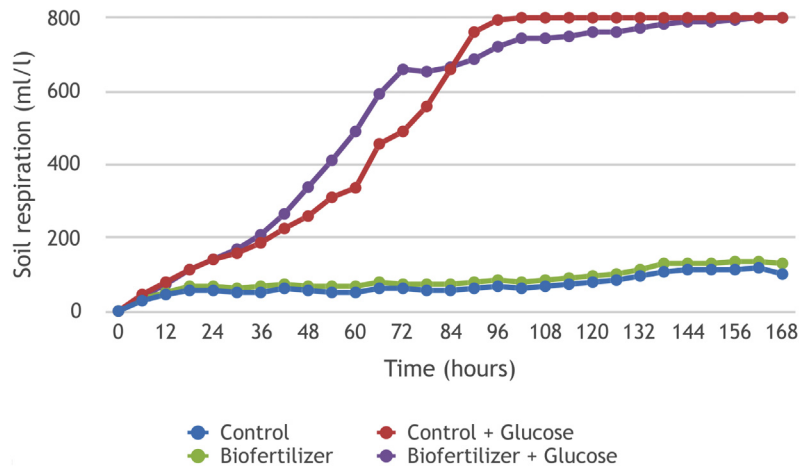
| Soil property                  | Value | Unit                   | Protocol   |
|--------------------------------|-------|------------------------|--|
| Silt and clay fraction         | 37.48 | %                      | Buzásl. (1988): Manual of Soil and Agrochemical Analysis Vol.1. (in Hungarian). INDA 4231 Kiadó. Budapest.   |
| Hygroscopicity                 | 2.23  | hy                     | Buzásl. (1988): Manual of Soil and Agrochemical Analysis Vol.1. (in Hungarian). INDA 4231 Kiadó. Budapest.   |
| Arany-type of plasticity limit | 39    | KA                     | Szegi  |
| Moisture content               | 19–21 | %                      | Szegi  |
| Hydrolytic acidity             | 5.94  | y1                     | Buzásl. (1988): Manual of Soil and Agrochemical Analysis Vol.1. (in Hungarian). INDA 4231 Kiadó. Budapest.   |
| organic-C                      | 1.4   | %                      | Székely  |
| Nitrate-N                      | 7.4   | mg/kg                  | Hayashi A, Sakamoto K, Yoshida T 1997: A rapid method for determination of nitrate in soil by hydrazine reduction produce. Jpn. J. Soil Sci. Plant Nutr.,68, 322 |
| Total-N                        | 2.6   | mg g <sup>-1</sup> D.S |  |
| AL-soluble P                   | 48.6  | P2O5 mg/kg             | Szegi  |
| AL-soluble K                   | 222   | K2O mg/kg              | Szegi  |
| pH (H2O)                       | 6.8   | pH                     | Buzásl. (1988): Manual of Soil and Agrochemical Analysis Vol.1. (in Hungarian). INDA 4231 Kiadó. Budapest.   |
| pH (KCl)                       | 6.1   | pH                     | Buzásl. (1988): Manual of Soil and Agrochemical Analysis Vol.1. (in Hungarian). INDA 4231 Kiadó. Budapest.   |
| Topsoil                        | 80–90 | cm                     | Szegi  |
| Soil texture                   | Loam  |                        | Buzásl. (1988): Manual of Soil and Agrochemical Analysis Vol.1. (in Hungarian). INDA 4231 Kiadó. Budapest.   |
| Minimal water capacity         | 26.22 | VKmin                  | Szegi  |
| Humus content                  | 2.81  | %                      | Buzásl. (1988): Manual of Soil and Agrochemical Analysis Vol.1. (in Hungarian). INDA 4231 Kiadó. Budapest.   |
| Total number of bacteria       | 9.59  | 1.000.000 colony/g     | Szegi  |
| Nitrate exploration            | 34.28 | mg/kg                  | Felföldy   |
| Microbial biomass carbon       | 333   | mg/kg                  | Vance ED, Brookes PC, Jenkinson DS 1987: An extraction method for measuring soil microbial biomass-C. Soil Biol. Biochem.,19, 703–707.                           |

## Results

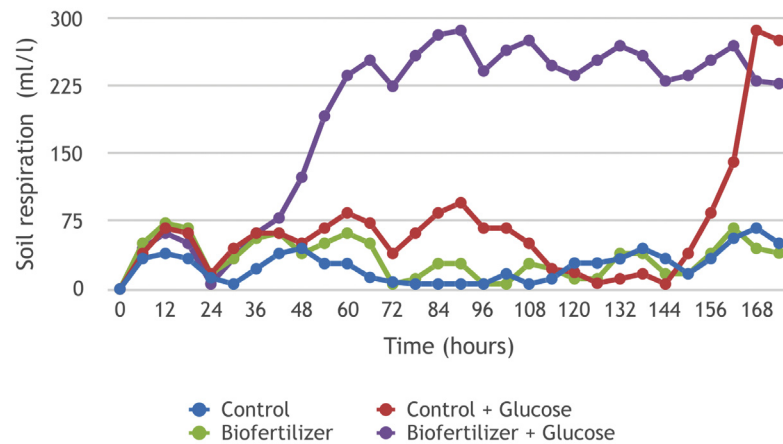
The treated samples produced more  $CO_2$  than the controls, as expected (Dataset 1). Each repeat with the exception of one showed increasing  $CO_2$  values (Figure 1), as the pressure continuously decreased in the bottle due to gas (oxygen) consumption. One sample produced unexpected results (Figure 2). In the first 12 hours, the treated samples produced more  $CO_2$  than the controls in each measurement. Following this, a fluctuation in the values was observed.

**Dataset 1. Average values of produced  $CO_2$  (ml/l) with different treatments 'Control' does not contain fertilizer, nor added glucose. 'Control+Glucose' contains 0,1 g of added glucose. 'Biofertilizer' contains Phylazonit biofertilizer. 'Biofertilizer+Glucose' contains Phylazonit biofertilizer and 0,1 g of added glucose**

<http://dx.doi.org/10.5256/f1000research.12936.d182663>



**Figure 1. Differences in CO<sub>2</sub> production of treated and control soil samples.** The induced method was carried out so that the difference between the results of control and the treated soil samples could become detectable sooner. Glucose was applied as inducer. As expected the CO<sub>2</sub> values increase or stagnate.



**Figure 2. This sample shows CO<sub>2</sub> values periodically decreasing in all conditions.** After examining the Oxitop device's operation, this pattern became more interesting to us, as the device quantifies CO<sub>2</sub> production by measuring BOD required for the degradation of organic matter. From the decreasing CO<sub>2</sub> values, we conclude that there was oxygen production and/or CO<sub>2</sub> consumption in the Oxitop bottles.

**Dataset 2. Comparison of produced CO<sub>2</sub> (ml/l) in the sample in which unexpected (periodically decreasing CO<sub>2</sub>) values can be observed. 'Control' does not contain fertilizer, nor added glucose. 'Control+Glucose' contains 0,1 g of added glucose. 'Biofertilizer' contains Phylazonit bio-fertilizer. 'Biofertilizer+Glucose' contains Phylazonit bio-fertilizer and 0,1 g of added glucose**

<http://dx.doi.org/10.5256/f1000research.12936.d182664>

## Discussion

In a closed system where the pressure decreases due to oxygen consumption, the values of CO<sub>2</sub> production must increase or stagnate with the passage of time, but this was not the case with

one of the samples (Figure 2). Here, a decrease in CO<sub>2</sub> occurred (Dataset 2). The following possible explanations were excluded:

- Presence of algae: there was no light in the incubator, so there was no photosynthesis.
- Changing pressure caused by changing temperature: the temperature was constant in the setup.
- Absorption by the water in the sample: all other samples that produced increasing amount of CO<sub>2</sub> had the same or comparable moisture content.

One reason that seemed more likely was that CO<sub>2</sub> oxidizing microbes or methanotrophs may have been present in the soil,

using the produced  $CO_2$  periodically. This is unusual, since most of the studies report the presence of these bacteria in seawater<sup>34</sup>, paddy fields<sup>35</sup> or industrial processes<sup>36</sup> and not in well-ventilated Chernozem soil. Further genomics research could detect the bacterial strains that consumed the  $CO_2$  in this soil.

### Data availability

Dataset 1: Average values of produced  $CO_2$  (ml/l) with different treatments. 'Control' does not contain fertilizer, nor added glucose. 'Control+Glucose' contains 0,1 g of added glucose. 'Biofertilizer' contains Phylazonit bio-fertilizer. 'Biofertilizer+Glucose' contains Phylazonit bio-fertilizer and 0,1 g of added glucose. DOI, [10.5256/f1000research.12936.d182663](https://doi.org/10.5256/f1000research.12936.d182663)<sup>37</sup>.

Dataset 2: Comparison of produced  $CO_2$  (ml/l) in the sample in which unexpected (periodically decreasing  $CO_2$ ) values can be observed. 'Control' does not contain fertilizer, nor added glucose.

'Control+Glucose' contains 0,1 g of added glucose. 'Biofertilizer' contains Phylazonit bio-fertilizer. 'Biofertilizer+Glucose' contains Phylazonit bio-fertilizer and 0,1 g of added glucose. DOI, [10.5256/f1000research.12936.d182664](https://doi.org/10.5256/f1000research.12936.d182664)<sup>38</sup>.

### Competing interests

No competing interests were disclosed.

### Grant information

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### Acknowledgements

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# Open Peer Review

Current Referee Status:  

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## Version 2

Referee Report 22 December 2017

doi:10.5256/f1000research.14500.r29290



**Muhammad Aslam Ali**

Department of Environmental Science, Bangladesh Agricultural University, Mymensingh, Bangladesh

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard to publish as a research note, however in case of a full manuscript it needs more scientific investigation on the variation of soil respiration as well as CO<sub>2</sub> fluxes and soil microbial activities under specified environmental conditions.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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## Version 1

Referee Report 29 November 2017

doi:10.5256/f1000research.14027.r27583



**Muhammad Aslam Ali**

Department of Environmental Science, Bangladesh Agricultural University, Mymensingh, Bangladesh

1. Why did the authors select Phylazonit biofertilizer? Does it contain any methanotrophs bacterial spp. or any electron acceptors? Didn't find the composition.
2. Why not investigate the CO<sub>2</sub> production rate with varying levels such as 0.5%, 1% and 5% substrates application in soils?
2. What were the initial content of organic carbon, total nitrogen, soil pH, redox status (Soil Eh) and microbial composition of the collected 24 soil samples?
3. How did the researchers control the pressure within the glass bottles during the experimental period?
4. How did the authors maintain moisture levels or water filled pore space uniformity in the 24 soil samples containing glass bottles?
5. Why didn't you collect the gas samples evolved from the soils in glass bottles at varying time hours?
6. Why didn't you follow the light/dark conditions in the Incubator where the glass bottles were kept?
7. All the Figures are not clear, no contrasting colors or bullets with lines used to differentiate the treatments.



8. How were soil microbial activities assessed? Methanogens and methanotroph's relative intensity were not found in this script, which are the major focus related to the current research topic.

**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Soil GHGs flux measurement, soil microbes & environment

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 01 Dec 2017

**Gabriela Bautista,**

Dear Prof. Muhammad Aslam Ali

We are trying to answer your questions, and submit a second version of the manuscript in order to clarify the following points.

**1. Why did the authors select Phylazonit biofertilizer? Does it contain any methanotrophs bacterial spp. or any electron acceptors? Didn't find the composition.**

The Phylazonit Ltd. provided the biofertilizer for test.

The Methods chapter begins with the information related to the composition: "*Bacillus megaterium*, *Bacillus circulans*, *Pseudomonas putida*, in an optimized ratio for soil injection". We did not say that the fertilizer contains methanotrophs bacterial spp. or any electron acceptors. That's why the results presented in the paper are unexpected.

**2. Why not investigate the CO<sub>2</sub> production rate with varying levels such as 0.5%, 1% and**

**5% substrates application in soils?**

This Research note discusses only unexpected results come from an experiment that was carried out using Oxitop devices. This is part of a project in which more methods are applied. In an other method (using liquid-alkaline absorption) is possible to setup the different levels, but that is not part of the discussion of the present paper. Using Oxitop bottles only one level is possible for the setup.

**2. What were the initial content of organic carbon, total nitrogen, soil pH, redox status (Soil Eh) and microbial composition of the collected 24 soil samples?**

In the Dataset 1: Average values for a number of different soil properties you can find the main physical, chemical and microbial soil properties such as pH (H<sub>2</sub>O), pH (KCl), Organic carbon. We will extend the dataset with the Total Nitrogen in the second version of the paper.

**3. How did the researchers control the pressure within the glass bottles during the experimental period?**

The Oxitop automatically measures the changes in the bottles due to gas consumption by its sensor, there is no needed to apply external measurement.

**4. How did the authors maintain moisture levels or water filled pore space uniformity in the 24 soil samples containing glass bottles?**

The measurement was carried out in closed system (bootles), it is not possible to open the bottles during the measurement.

**5. Why didn't you collect the gas samples evolved from the soils in glass bottles at varying time hours?**

The Oxitop continously measures the changes. As Fig.1 and Fig.2 show during 168 hours the gas oxygen consumption/ CO<sub>2</sub> production were measured.

**6. Why didn't you follow the light/dark conditions in the Incubator where the glass bottles were kept?**

In order to avoid the effect of the photosynthesis by algaeas. We were interested in soil bacteria activities only.

**7. All the Figures are not clear, no contrasting colors or bullets with lines used to differentiate the treatments.**

We do not understand this question. In Both figures we used different colors and bullets.

- Control (absolute): Blue
- Control + glucose: Red
- Treated: Green
- Treated + glucose: Purple

**8. How were soil microbial activities assessed? Methanogens and methanotroph's relative intensity were not found in this script, which are the major focus related to the current research topic.**

This paper was submitted as a Research note. Research notes are often *preliminary studies*, descriptions of unexpected and perhaps unexplained observations or lab protocols. We concluded that "Further genomics research could detect the bacterial strains that consumed the CO<sub>2</sub> in this soil."

**Gabriela Bautista, Bence Mátyás**

**Competing Interests:** No competing interests were disclosed.

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**Ankit Singla**

Regional Centre of Organic Farming, Bhubaneswar, Odisha, India

Bautista and Matyas observed unexpected results in Chernozem soil respiration following the different fertilizer treatments. I think, the values of Dataset 2 could be directly included in the main content of the paper, if possible. The title of Dataset 1 should be "Average values for various properties of Chernozem soil".

I have answered 'partly' to the question 'Are all the source data underlying the results available to ensure full reproducibility?' as soil ecosystems are very diverse and results could vary under different environmental conditions.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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