Unexpected results in Chernozem soil respiration while measuring the effect of a bio-fertilizer on soil microbial activity [version 1; peer review: 1 approved, 1 approved with reservations]

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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 CO2 values while examining Chernozem soil samples. We concluded that CO2 oxidizing microbes or methanotrophs may be present in the soil that periodically consume CO2. This is unusual for a sample taken from the upper layer of well-ventilated Chernozem soil with optimal moisture content.

Keywords
bio-fertilizer, soil respiration, Chernozem, OxiTop
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Introduction
Research related to the benefits of microbes as biofertilizer has become increasingly important in the agricultural sector. This is due to their potential role in 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. Soil respiration is an important indicator of soil microbial activity. In our experiments, we measured the effect of different chemicals and a biofertilizer 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
The phylazonit bio-fertilizer (produced by Phylazonit.Ltd, Hungary) was used for testing (15 l/ha). It has the following composition: Bacillus megaterium, Bacillus circulans, Pseudomonas putida, in an optimized ratio for soil injection. Number of bacteria: 10^9 piece/cm^3.

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). All samples had optimum moisture content with 19–21 percent. Soil moisture content was determined gravimetrically by drying the soil at 105 degrees C for 24 hours according to Klimes-Szmik, 1970. The experimental design was completely randomized, treatments were applied at 25 °C. An OxiTop OC110 respirometer was used to quantify the release and capture of CO_2, 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 protocol in the manual (https://www.wtw.com/en/service/downloads/operating-manuals.html).

10g of soil sample were placed into OxiTop flasks, capped with the sensor heads according to Barrales-Brito et al., 2014. 2.5 g of CO_2 absorber (sodalime) were then added to a tank, to absorb the generated CO_2 according to Barrales-Brito et al., 2014. Induced samples were given 0.1g of glucose. 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_2 production.

Results
The treated samples produced more CO_2 than the controls, as expected. Each repeat with the exception of one showed growing 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.
Discussion

In a closed system where the pressure decreases due to oxygen consumption, the values 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 CO₂ occurred. The following possible reasons 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 CO₂ had the same or comparable moisture content.

One reason that seemed more likely was that CO₂ oxidizing microbes or methanotrophs may have been present in the soil, periodically using the produced CO₂. This is unusual since most of studies report the presence of these bacteria in seawater15, paddy fields16 or industrial processes17 and not in well-ventilated Chernozem soil. Further genomics research could detect the bacterial strains that consumed the CO₂ in this soil.

Data availability

Dataset 1: Average values for a number of different soil properties. DOI, 10.5256/f1000research.12936.d1826515.

Dataset 2: Average values of produced CO₂ (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.d18266316.

Dataset 3: Comparison of produced CO₂ (ml/l) in the sample in which unexpected (periodically decreasing CO₂) 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.d18266417.

Competing interests

No competing interests were disclosed.

Grant information

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Acknowledgements

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Version 1

Reviewer Report 29 November 2017

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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 CO2 production rate with varying levels such as 0.5%, 1% and 5% substrates application in soils?
3. 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?
4. How did the researchers control the pressure within the glass bottles during the experimental period?
5. How did the authors maintain moisture levels or water filled pore space uniformity in the 24 soil samples containing glass bottles?
6. Why didn’t you collect the gas samples evolved from the soils in glass bottles at varying time hours?
7. Why didn’t you follow the light/dark conditions in the Incubator where the glass bottles were kept?
8. All the Figures are not clear, no contrasting colors or bullets with lines used to differentiate the treatments.
9. 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.

Reviewer 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, University of Debrecen, Debrecen, Hungary

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 CO2 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 (H2O), 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
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 (bottles), 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 continuously measures the changes. As Fig.1 and Fig.2 show during 168 hours the gas oxygen consumption/ CO2 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 algae. 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 CO2 in this soil."

Gabriela Bautista, Bence Mátyás

**Competing Interests:** No competing interests were disclosed.
Ankit Singla
Regional Centre of Organic Farming, Ministry of Agriculture and Farmers Welfare, Government of India, 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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