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Short communication

A new protocol for an artificial soil to analyse soil microbiological processes

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ABSTRACT

Soils are highly complex environments characterized by a huge diversity of organisms (especially microorganisms) and chemical compounds and by complex physical structure. Because of this complexity, it is often difficult to manipulate independently the microbial community, the organic matter, the clay types, etc., and to disentangle the various processes involved in soil functioning. In this paper, we propose the use of artificial soils as a simplified and adjustable tool to disentangle soil processes and test ecological theories on microbial communities. To create an artificial soil, a protocol was designed based on commercially available clays, sand, calcium carbonates and humic acids. Special attention was paid to aggregates and structure formation using differential sieving. Many aspects of our artificial soil can be adjusted as needed by altering mineral nutrient or humic acid concentrations, addition of other organic molecules, varying the quality of clays, etc. The advantage of an artificial soil is that chemical and biological diversity as compared with real soils can be reduced so that the effects of manipulations (adding an organism or a molecule, changing temperature, etc.) are not confounded by uncontrolled interactions. To test the capability of this artificial soil to support microbial growth and dynamics, six bacterial strains were independently inoculated and monitored for 19 days. Each strain was able to grow and mineralize the available organic matter. This artificial soil could thus be a good tool for studying different aspects of soil functioning.

1. Introduction

During recent decades, the development of molecular biology tools has greatly improved our knowledge of microbial diversity. The huge microbial diversity of the soil has led ecologists to develop new ways of studying soil functioning (Fontaine et al., 2003). Soils are highly complex environments due to the diversity of mineral and organic compounds, their physico-chemical structure (Whalley et al., 2005; Hinsinger et al., 2009) and the highly heterogeneous distribution of the resources: water, mineral nutrients, organic matter, and oxygen (Ilstedt et al., 2006; Gregory et al., 2007). Consequently, soil complexity makes it difficult to deeply understand the different ecological processes.

One possible alternative approach could involve using simplified experimental systems able to reproduce selected properties for further studies. In soil, several authors have considered that simplified experimental systems could allow testing alternative ecological hypotheses (Barot et al., 2007; Prosser et al., 2007). Soil microcosms are an example of simplified systems which have been widely used for many years, especially for controlling the presence of various soil organisms (Bonkowski and Roy, 2005; Laossi et al., 2009). However, due to their physical, chemical and biological complexity, it is difficult to manipulate soil characteristics independently and understand how they interact. For example, the study of the direct influence of clays on soil C storage through adsorption of soil organic matter (SOM) requires the comparison of the C turnover in different soils containing different types and proportions of clays. However, clay types and their proportions might change the microbial enzyme adsorption (Quiquampoix et al., 2002) and indirectly influenced SOM mineralization. Thus, direct and indirect effects of clay types and proportions might interfere making difficult the clear identification of the clay effects. Moreover, due to the high microbial diversity in natural soils, it is difficult to identify the microorganisms involved in particular aspects of soil functioning such as mineralization or priming effects (Blagodatskaya and Kuzyakov, 2008). It is also difficult to assess the respective influence of physico-chemical and biological limitations of mineralization (Kemmitt et al., 2008). Manipulating independently these parameters in an artificial soil could help to understand how they interact. In this respect, the problem is that when manipulating soil fractions coming from natural soils, it is impossible not to manipulate the organic matter, and the microorganisms contained in these soil

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Table 1
Artificial soil characteristics.

C content ($mg\,g^{-1}$)	N content ($mg\,g^{-1}$)	C:N ratio	pH	Soil bulk density ($g\,cm^{-3}$)	Aggregates bulk density ($g\,cm^{-3}$)	Soil pore volume ($cm^{-3}\,g^{-1}$)	Microporosity, ($cm^{-3}\,g^{-1}$)	Macroporisty ($cm^{-3}\,g^{-1}$)	Clay content (%)	Sand content (%)	CEC ($cmol+\,kg^{-1}$)
8.6 (0.1)[a]	0.15 (0.01)	56.0 (2.1)	9.9	0.96	1.6	0.67	0.25	0.42	29.4	68.5	5.7 (0.2)

[a] Numbers in parentheses are standard deviation.

fractions (Bronick and Lal, 2005). Conversely, sterilizing the soil and inoculating a limited number of bacterial species leaves the soil with an unrealistic quantity of dead organic matter resulting from the death of bacteria. It is still a challenge to propose experimental systems that simplify soil characteristics, and yet maintain its functioning. We suggest that using artificial soils with improved control of the physical, chemical and biological characteristics could help in reaching this goal.

Creating artificial soils to study microbiological processes is an old quest (Madhok, 1937). Moreover, several protocols exist such as that used in ecotoxicology (OECD, 2004) but to our knowledge the organic matter used in these protocols is peat. Consequently, the carbon source and the soil C:N ratio or the microbial community could not be easily controlled. One of the first easily adjustable protocols with a realistic organic matter source to make an artificial soil was proposed by Ellis (2004). It consisted of a simple mix of clays, sand, humic acids and calcium carbonate without any control of soil structure or mineral nutrient content, despite the importance of these two factors for soil functioning (Bossuyt et al., 2001; Ball and Robertson, 1994; Craine et al., 2007; Stark et al., 2007; Alvaro-Fuentes et al., 2009). To address these shortcomings we propose an improved protocol, in which addition of mineral nutrients and creation of aggregates was possible.

2. Materials and methods

2.1. The artificial soil

The different steps of the protocol consist in mixing the elementary components, and creating and wetting the aggregates. Firstly, kaolinite (20 g, Sigma, Poole, UK), a 1/1 clay type with low Cation Exchange Capacity ($2.8 \pm 0.1\,cmol+\,kg^{-1}$), was mixed with bentonite (10 g, Sigma, Poole, UK), a 2/1 clay type with high CEC ($59.0 \pm 0 = 1.0\,cmol+\,kg^{-1}$). Bentonite has swelling/shrinking behaviour during wetting and drying cycles unlike kaolinite. Then, distilled water (300 ml) was added and the suspension was agitated for two days at 125 rpm. Afterwards, in order to avoid clay microaggregate formation, the suspension was sonicated (ultrasonicator 300 Ultrasonik, Ney, New York, USA) at maximal power for 10 min. Then, the suspension was centrifuged ($200 \times g$, 10 min) and the precipitate was collected and stored at 4 °C before use.

Secondly, 70 g of sand were mixed with the precipitate containing 30 g of clay with a spatula until a uniform 'mud' was obtained after approximately 10 min. The sand was pure quartz (Fontainebleau sand) and particle diameter ranged from 50 to 2000 μm with a majority of small particles (<200 μm).

Finally, to avoid heterogeneous drying with cracks separating wet clods, the 'mud' was spread over a glass micro-fibre filter GF/D of 2.7 μm (Whatman, Maidstone, UK) covered with a 25 μm mesh at 4 °C for three days. This drying process resulted in a cake that was gently broken into small pieces. These aggregates were sieved at 4 and 0.500 mm and we collected only the aggregates ranging from 500 μm to 4 mm.

To mimic the organic carbon contained in soils, humic acids (2 g, Acros, Loughborough, UK) were added to the clays/sand aggregates (98 g). Then, to reduce acidity, 0.2 g of $CaCO_3$ (Acros, Loughborough, UK) was also added. Finally, the artificial soil was sterilized in sealed plastic bags by γ-irradiation at 50 kGy (Ionisos, Dagneux, France) and stored at room temperature until use (see supplementary material in online version). Prior to incubation, a fraction of the soil (20 g) was remoistened with 2.5 ml of COMBO medium in sterile glass flasks for three days (Kilham et al., 1998), to allow the water to be homogeneously distributed. The regular brown colour of the aggregates (see supplementary material in online version) confirms that humic acid was homogenously distributed within soil aggregates.

Aggregate sizes were still distributed between 500 μm and 4 mm. After sieving, aggregates showed a cubic form, due to the geometry of the sieve mesh; after remoistening, their shape was more rounded and thus more similar to real soils aggregates. This wetting method, although very simple (no control of wetting rate), was good enough to avoid aggregate slaking and destruction, and to provide a realistic aggregate shape and consequently a realistic geometry of packing pores (see supplementary material in online version). The main characteristics of the artificial soil are summarised in Table 1.

2.2. Microbial growth

Six bacterial strains were inoculated: *Pseudomonas fluorescens* (DSMZ 7153), *Massilia lutea* (DSMZ 17473), *Variovorax paradoxus* (DSMZ 30034), *Rhizobium radiobacter* (DSMZ 9674), *Hyphomicrobium facile* (DSMZ 1565) and *Ramlibacter henchirensis* (CIP 108694). These strains were selected because they were isolated from different soil types in different ecosystems and are known to present a large range of ecological characteristics, and particularly to have different capacities in using humic acid as a growth substrate (Atlas and Bartha, 1998; Sarathchandra et al., 1997; Padmanabhan et al., 2003; Hashimoto et al., 2006; Bernard et al., 2007). Prior to inoculation into the artificial soil, bacteria were cultivated according to manufacturer's instructions and transferred into a sterilized humic acid solution. They were maintained in growth phase by regular inoculation in sterilized humic acid solution. To inoculate the bacteria and to avoid excessive and non-controlled input of humic acids, bacteria were concentrated by centrifugation and then diluted in COMBO medium before inoculation into the soil (0.5 ml corresponding to a total COMBO addition of 3 ml). The final concentration of bacteria inoculated was 6×10^6 cells/g of dried soil. The experimental units consisting of 20 g of dried artificial soil remoistened with COMBO (15%, w/w) placed into a 120 ml flask and subsequently sealed with a septum. A control treatment, with only COMBO addition, was performed to check for the artificial soil sterility. Each culture was performed in triplicate and incubated for 19 days at 25 °C. At the beginning of the experiment, the flask's atmosphere was CO_2-free. Bacterial counts were performed on TSA plates (Tryptone Soja Agar, Fluka Chemicals, Castel Hill, Australia) after 24 h at room temperature. The CO_2 concentration was measured using a MICROGC (Agilent, Santa Clara, USA), and the flask was flushed with reconstituted and CO_2-free air. Bacterial counts and CO_2 measurements were performed after 2, 4, 6, 8, 10 and 19

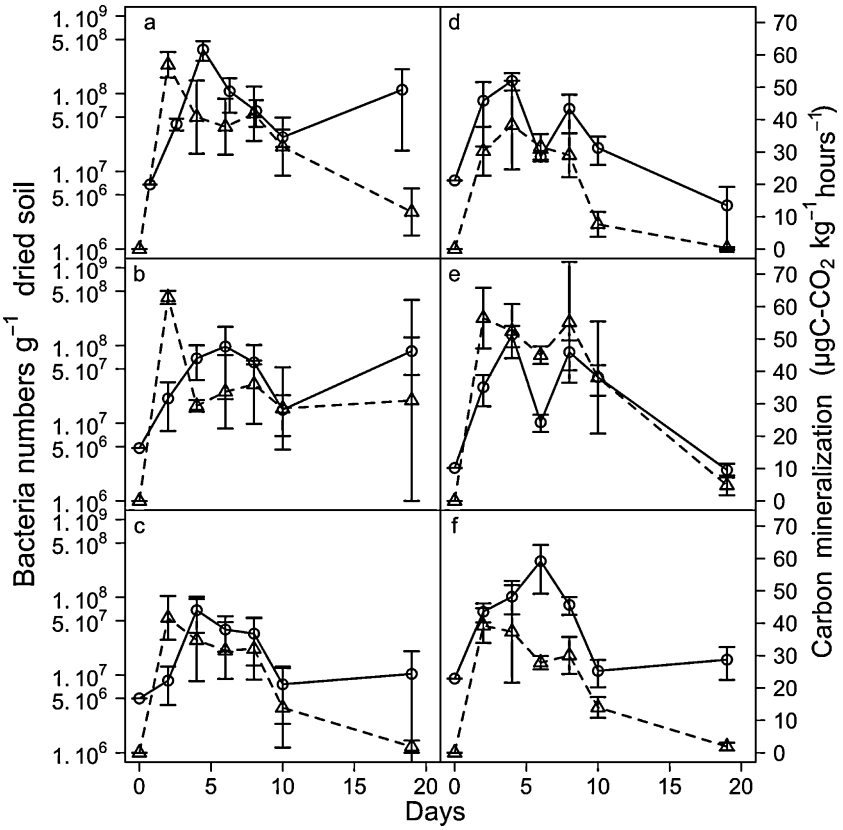

Fig. 1. Bacterial growth curves (○) and carbon mineralization(△) for the six strains: (a) *Hyphomicrobium facile* (DSMZ 1565), (b) *Massilia lutea* (DSMZ 17473), (c) *Ramlibacter henchirensis* (CIP 108694), (d) *Rhizobium radiobacter* (DSMZ 9674), (e) *Variovorax paradoxus* (DSMZ 30034), (f) *Pseudomonas fluorescens* (DSMZ 7153), growth curves were assessed in triplicates (mean ± s.e., $n = 3$).

days of incubation. The amount of CO_2 produced by carbonate acidification was estimated in the control treatment and subtracted from the amount of CO_2 measured within the flasks containing inoculated soil.

All statistical analyses were performed using R (version 2.7.1) (R Development Core Team, 2008). Linear mixed effect models were performed with times and strains as fixed variables and replicates as a random effect. Then, a post hoc Tukey test was performed to detect difference within the treatments. Because bacterial count data distribution was neither normal nor homosedastic, they were log transformed.

3. Results and discussion

Fig. 1 shows the growth of each bacterial strain in the artificial soil. We observed a significant increase in bacterial density and CO_2 production during the experiment for each strain ($p < 0.001$), indicating that all the strains were able to grow and to mineralize humic acids. Similar dynamics were observed for all strains except for *R. henchirensis* that had a maximal bacterial density lower than *H. facile* ($p < 0.0001$) and *P. fluorescens* ($p < 0.001$). These differences could be due to differences in the efficiency of using humic acids as a carbon source. Although some bacterial cells might have died after inoculation and created a carbon source, the dead microbial biomass was probably not large enough to support the growth observed. These results suggest that the six strains were able to use the humic acids incorporated into the soil.

Our results show that our artificial soil was indeed a favourable environment with no physical limiting factor for the development of different bacterial strains. The soil structure resulting from the packing of artificial aggregates created a sufficient pore size distribution including intra- and inter-aggregates pores, as exists in natural soils. This approach should be useful to better understand the effect of soil parameters (texture, structure, moisture, pH, microbial communities structure, SOM quantity, quality and diversity) and their interactions on emergent soil properties such as organic matter dynamics, the release of mineral nutrients, total microbial biomass, trophic interactions, etc. Moreover, the artificial soil designed in this study might also be useful to study plant–microbial interactions. We could, for example, remove the humic acids and study the direct effect of plant exudation as sole carbon source, on the microbial community structure.

4. Conclusion

Our protocol was successful in creating an artificial soil with a structure able to mimic natural soil, stable when water was added and allowing bacterial development. Consequently, the structuring and humidification protocols as well as the types and the proportions of the minerals used were well adapted to our requirements. The tool proposed in this paper is modular and very flexible: it can be adjusted both to reproduce more features of real soils and to modify one by one those key features to test their influence. Further tests are needed to evaluate this soil as a substrate for a wider range of soil biota including other bacteria, fungi, earthworms, and plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.apsoil.2011.04.002.

References

Alvaro-Fuentes, J., Cantero-Martinez, C., Lopez, M.V., Paustian, K., Denef, K., Stewart, C.E., Arrue, J.L., 2009. Soil aggregation and soil organic carbon stabilization: effects of management in semiarid Mediterranean agroecosystems. Soil Sci. Soc. Am. J. 73, 1519–1529.

Atlas, R.M., Bartha, R., 1998. Microbial Ecology: Fundamentals and Applications, 4th ed. The Benjamin/Cummings Publishing Co. Inc.

Ball, B.C., Robertson, E.A.G., 1994. Soil structure and transport-properties associated with poor growth of oilseed rape in soil direct drilled when wet. Soil Till. Res. 31, 119–133.

Barot, S., Blouin, M., Fontaine, S., Jouquet, P., Lata, J., Mathieu, J., 2007. A tale of four stories: soil ecology, theory, evolution and the publication system. PLoS One, 2.

Bernard, L., Mougel, C., Maron, P., Nowak, V., Lévêque, J., Henault, C., Haichar, F.E., Berge, O., Marol, C., Balesdent, J., Gibiat, F., Lemanceau, P., Ranjard, L., 2007. Dynamics and identification of soil microbial populations actively assimilating carbon from ^{13}C-labelled wheat residue as estimated by DNA- and RNA-SIP techniques. Environ. Microbiol. 9, 752–764.

Blagodatskaya, E., Kuzyakov, Y., 2008. Mechanisms of real and apparent priming effects and their dependence on soil microbial biomass and community structure: critical review. Biol. Fertil. Soils 45, 115–131.

Bonkowski, M., Roy, J., 2005. Soil microbial diversity and soil functioning affect competition among grasses in experimental microcosms. Oecologia 143, 232–240.

Bossuyt, H., Denef, K., Six, J., Frey, S.D., Merckx, R., Paustian, K., 2001. Influence of microbial populations and residue quality on aggregate stability. Appl. Soil Ecol. 16, 195–208.

Bronick, C.J., Lal, R., 2005. Soil structure and management: a review. Geoderma 124, 3–22.

Craine, J.M., Morrow, C., Fierer, N., 2007. Microbial nitrogen limitation increases decomposition. Ecology 88, 2105–2113.

Ellis, R., 2004. Artificial soil microcosms: a tool for studying microbial autecology under controlled conditions. J. Microbiol. Methods 56, 287–290.

Fontaine, S., Mariotti, A., Abbadie, L., 2003. The priming effect of organic matter: a question of microbial competition? Soil Biol. Biochem. 35, 837–843.

Gregory, A.S., Watts, C.W., Whalley, W.R., Kuan, H.L., Griffiths, B.S., Hallett, P.D., Whitmore, A.P., 2007. Physical resilience of soil to field compaction and the interactions with plant growth and microbial community structure. Eur. J. Soil Sci. 58, 1221–1232.

Hashimoto, T., Whang, K., Nagaoka, K., 2006. A quantitative evaluation and phylogenetic characterization of oligotrophic denitrifying bacteria harbored in subsurface upland soil using improved culturability. Biol. Fertil. Soils 42, 179–185.

Hinsinger, P., Bengough, A.G., Vetterlein, D., Young, I.M., 2009. Rhizosphere: biophysics, biogeochemistry and ecological relevance. Plant Soil 321, 117–152.

Ilstedt, U., Nordgren, A., Malmer, A., 2006. Soil chemical and microbial properties after disturbance by crawler tractors in a Malaysian forest plantation. Forest Ecol. Manag. 225, 313–319.

Kemmitt, S.J., Lanyon, C.V., Waite, I.S., Wen, Q., Addiscott, T.M., Bird, N.R.A., O'Donnell, A.G., Brookes, P.C., 2008. Mineralization of native soil organic matter is not regulated by the size, activity or composition of the soil microbial biomass—a new perspective. Soil Biol. Biochem. 40, 61–73.

Kilham, S.S., Kreeger, D.A., Lynn, S.G., Goulden, C.E., Herrera, L., 1998. COMBO: a defined freshwater culture medium for algae and zooplankton. Hydrobiologia 337, 147–159.

Laossi, K.-L., Noguera, D.C., Bartolomé-Lasa, A., Mathieu, J., Blouin, M., Barot, S., 2009. Effects of endogeic and anecic earthworms on the competition between four annual plants and their relative reproduction potential. Soil Biol. Biochem. 41, 1668–1773.

Madhok, M.R., 1937. Synthetic soil as a medium for the study of certain microbiological processes. Soil Sci. 44, 319–322.

OECD, 2004. Guideline for Testing of Chemicals No. 222, Earthworm Reproduction Test (*Eisenia fetida*/*andrei*). Organization for Economic Co-operation and Development, Paris, France.

Padmanabhan, P., Padmanabhan, S., DeRito, C., Gray, A., Gannon, D., Snape, J.R., Tsai, C.S., Park, W., Jeon, C., Madsen, E.L., 2003. Respiration of 13C-labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of 13C-labeled soil DNA. Appl. Environ. Microbiol. 69, 1614–1622.

Prosser, J.I., Bohannan, B.J., Curtis, T.P., Ellis, R.J., Firestone, M.K., Freckleton, R.P., Green, J.L., Green, L.E., Killham, K., Lennon, J.J., Osborn, A.M., Solan, M., van der Gast, C.J., Young, P., 2007. The role of ecological theory in microbial ecology. Nat. Rev. Microbiol. 5, 384–392.

Quiquampoix, H., Servagent-Noinville, S., Baron, M.-H., 2002. Enzyme adsorption on soil mineral surfaces and consequences for the catalytic activity. In: Burns, R.G., Dick, R.P. (Eds.), Enzymes in the Environment. Marcel Dekker, New York, pp. 285–306.

R Development Core Team, 2008. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Sarathchandra, S.U., Burch, G., Cox, N.R., 1997. Growth patterns of bacterial communities in the rhizoplane and rhizosphere of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) in long-term pasture. Appl. Soil Ecol. 6, 293–299.

Stark, C., Condron, L.M., Stewart, A., Di, H.J., O'Callaghan, M., 2007. Influence of organic and mineral amendments on microbial soil properties and processes. Appl. Soil Ecol. 35, 79–93.

Whalley, W.R., Riseley, B., Leeds-Harrison, P.B., Bird, N.R.A., Leech, P.K., Adderley, W.P., 2005. Structural differences between bulk and rhizosphere soil. Eur. J. Soil Sci. 56, 353–360.