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Original Article

Characterization of Microbial Community in Selected Mineral Soils after Long-Term Storage

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A. Wolińska[1*]
Z. Stępniewska[1]
A. Wołoszyn[1]

1- The John Paul II Catholic University of Lublin, Institute of Biotechnology, Department of Biochemistry and Environmental Chemistry, Al. Kraśnicka 102, 20-718 Lublin, Poland*
Email: awolin@kul.lublin.pl

Abstract

It is a common practice to store soil samples before measurements in a lab as an alternative to *in-situ* studies. Soil banks contain large variety of soil types, some of which may have a long shelf-life. Thus it is desirable to explore the effect of long-term storage on soil quality, particularly in terms of microbiological properties. Here, a 19-year storage period under air-dry conditions at 4ºC was selected for studying its effect on microbial presence and potential recovery after rewetting. The soil samples represented the top layer (0-30 cm) and had following FAO classifications: *Mollic Gleysol*, *Eutric Cambisol*, *Rendzina Leptosol*, *Orthic Podzol* and *Eutric Fluvisol* were used in the experiment. It was found that a 10 day incubation of aged soil under full water-capacity conditions and room temperature was sufficient for the microbial regeneration. Moisture content was determined for a range of water potentials (pF): 0, 1.5, 2.2 2.7 and 3.2, which represented different water availabilities for microorganisms and plant. It appeared that soil moisture content had significant effect (p<0.001) on the total number of bacteria and the most probable number (MPN) of ammonia oxidizing bacteria (AOB). Molecular analysis (16S rRNA) showed the dominance of *Betaproteobacteria* genera with the main representatives of *Nitrosomonas*, *Nitrosospira, Delftia*, *Comamonas*, *Pseudomonas*, and exponent species of *Firmicutes* genera such as *Clostridium* and *Ruminococcus*.

Keywords: *16S rRNA gene analysis, Soil water potential, Microorganisms abundance, Soil rewetting.*

INTRODUCTION

Soil is a complex and dynamic biological system, making it difficult to determine the composition of microbial communities in soil despite recent technological advances (Nannipieri et al., 2003). Interestingly, one gram of soil may harbor up to 10 billion microorganisms of possibly thousands of different species (Schloss and Handelsmann, 2006; Torvsik and Øvreås, 2002). As less than 1% of species of soil bacteria have been brought to laboratory conditions (Janssen, 2006; Schloss and Handelsmann, 2006), soil ecosystems remain, to a large extent, uncharted. Life in the soil environment is continuously subjected to drying and rewetting cycles. Liesack et al., (2000) reported that alternate flooding and drainage of the soils caused spatial and temporal changes of soil microbial communities and processes. Water is a critical resource and its availability impacts microbial activity within the soil matrix, thus periods of water limitation may affect microbial communities through starvation (Gleeson et al., 2008). Rapid rewetting of a dry soil causes microorganisms to undergo osmotic shock, possibly inducing cell lysis and a release of intracellular solutes (Gleeson et al., 2008; Iovieno and Bååth, 2008). Most anaerobic microbes form spores or resting stages, whereas asporogenous facultative anaerobes are able to modify metabolism in order to withstand oxidative stresses (Das and Dangar, 2008; Gleeson et al., 2008). Evidently, about 34% anaerobic bacteria can survive up to 2 years in the dry (oxic) period of the flooded soils (Liesack et al., 2000). According to Gleeson et al., (2008) AOB and nitrifying bacteria are the microbial functional groups influenced by a variety of environmental factors, including water content, that dictate

community parameters (i.e., number counts, diversity and activity *in situ*). Gleeson et al., (2008) noted also that AOB are well adapted to surviving extreme drought and become active within minutes of rewetting dry soils. These observations were the reason for undertaking the current study focused on determination AOB and nitrifying groups of soil microorganisms.

As soil is a very heterogeneous system comprising a variety of microhabitats with different physicochemical gradients and discontinuous environmental conditions (Grundmann et al., 2001; Nannipieri et al., 2003; Torvsik and Øvreås, 2002), soil microorganisms must adapt to these microhabitats and live together in consortia with more or less sharp boundaries, interacting with each other and with other biota. Bacterial activities have been reported to be unevenly distributed in soil, which led to the concept of hot spots linked to locally transient carbon available for microbial growth and activity (Grundmann et al., 2001; Nannipieri et al., 2003). Hot environments are within the life-supporting extreme niches that appear to have maintained some degree of special biotechnological interest (Grundmann et al., 2001; Tomova et al., 2010). The two main locations for active bacteria are believed to be in soil pores within surrounding water film, either in regions of preferential flow, or entrapped within the soil matrix (Grundmann et al., 2001). Analysis of the spatial distribution of bacteria at microhabitat levels demonstrated that more than 80% of the bacteria were located in micropores of 2-20 μm size constituting stable micro-aggregates (Sey et al., 2008; Torvsik and Øvreås, 2002). Such microhabitats offer the most favorable conditions for microbial growth with respect to water and substrate availabilities, gas diffusion and protection against predation (Torvsik and Øvreås, 2002). In addition, a high diversity of bacteria belonging to the *Acidobacterium* division and *Prosthecobacter* was identified in small particles, whereas large particles harbored microorganisms belonging to the *Alfaproteobacteria*.

Over the past two decades, molecular methods, especially 16S rRNA gene sequencing, have become very popular to help in identification of unknown bacteria (Janssen, 2006; Nannipieri et al., 2003; Torvsik and Øvreås, 2002). As a result, this has led to using an analysis of the total community DNA extracted from the environment. Fingerprinting techniques based on polymerase chain reaction (PCR) can offer high resolution and provide information about changes in the whole community structure (Torvsik and Øvreås, 2002). However, knowledge on biodiversity in terrestrial conditions is still scanty. There is also lack of investigations concentrated on biological life in the soil after long storage time (e.g. 20 years). One question is whether the biological life in such soils is still present; another is whether microbial spores have a potential to be active after prolonged storage if dormancy-type of a survival mechanism is possible. The twin objectives of the study were: (1) determination of which groups of soil microorganisms (AOB, nitrifying and general bacteria) are activated first after the long period of storage in response to soil rewetting, and (2) quantification of microorganisms under different water-content conditions.

MATERIAL AND METHODS

Soil description

The soil samples were obtained from the Bank of Polish Mineral Soil Samples (Institute of Agrophysics, PAS Lublin, Poland), established in 1991. The topsoil (0-30 cm) of *Mollic Gleysol* (Kolno, 22°42'E, 52°28'N), *Eutric Cambisol* (Tarnowo, 16°44E, 52°27'N), *Rendzina Leptosol* (Bezek, 23°20'E, 50°51'N), *Orthic Podzol* (Kolnica, 17°20'E, 50°45'N) and *Eutric Fluvisol* (Zawadka, 21°23'E, 49°54'N) were used in the experiments. All soils had been collected in Poland. The basic soil characteristics is presented in Table 1.

Table 1. Main features of investigated soil materials.

Type of soil	Bank No.	Granulometric composition (%)							Bulk density (g/cm^3)	pH (in H_2O)	C org. (%)
		Rock+ gravel	1-0.1 (mm)	0.1-0.05 (mm)	0.05-0.02 (mm)	0.02-0.005 (mm)	0.005-0.002 (mm)	Loam			
Mollic Gleysol	208	4	51	17	10	12	5	3	1.43	7.58	1.43
Eutric Cambisol	308	8	64	12	8	7	3	0	1.55	6.25	0.47
Rendzina Leptosol	563	6	49	7	18	14	5	4	1.40	7.95	0.89
Orthic Podzol	701	4	60	11	5	6	4	12	1.43	7.34	1.06

Eutric Fluvisol	967	0	17	14	25	18	14	12	1.25	5.98	1.41

Determination of soil water retention

Soil samples were collected using plastic containers (height of 4.5 cm, diameter of 2.9 cm), and pre-incubated through flooding in 20ºC for 10 days. Next the samples were placed in an airtight chamber, part of a laboratory set LAB o12 (Soil Moisture Equipment Company, USA), before a pressure was applied. The instrument for determining water curves is a steel pressure chamber, inside of which a porous plate saturated with water is located. At the bottom, soil samples, continuously exposed to atmospheric pressure, make the hydraulic contact with the porous plate (Pires et al., 2005).

The chamber is closed and the desired air pressure *P* is applied, driving away the soil water retained at pressures below *P,* until equilibrium is reached (Pires et al., 2005). The moisture content was determined via the drying process, for the range of water potentials (e.g., 0, 1.5, 2.2, 2.7 and 3.2 pF), corresponding to the amount of water available for microorganisms and plant roots as in the natural environment.

Microbial abundance

The soil samples (5 g each, with pF value: 0; 1.5 and 3.2) were suspended separately in 50 ml 0.85% NaCl. The colony form unit (CFU) of the total heterotrophic bacteria was calculated after 14 days of growth on an agar medium at 25°C. The AOB were counted after 7 days of incubation on water-peptone medium (Merck) at 26°C, whereas the nitrifying bacteria community was cultured on Winogradsky's medium and the colonies were visualized after 14 days, by flooding the tubes with sulphanillic acid reagent (8 g/l sulphanillic acid, 5 M acetic acid, and a 1:1 mixture by volume of 5 g/l α-naphthayl amine and 5 M acetic acid) which was responsible for the pink color of these bacteria. The compositions of media used in the study are summarized in Table 2. Population of microbes growing on solid mediums was expressed as CFU/g dry soil, and as MPN/g dry soil on liquid media.

Table 2. Composition of culture media used in the current experiment.

Soil organisms	Medium	Content (g/l)	
Total bacteria	Nutritive agar	Peptone	5.0
		Beef extract	3.0
		Agar-agar	12
Ammonia oxidizing bacteria	Peptone water (buffered)	Peptone from casein	10
		NaCl	5.0
		Na_2HPO_4	1.5
		KH_2PO_4	9.0
		Distilled water	1.0
Nitryfying bacteria	Vinogradsky medium	$(NH_4)_2SO_4$	2.0
		K_2HPO_4	1.0
		$MgSO_4 \cdot 7H_2O$	0.5
		NaCl	2.0
		$FeSO_4 \cdot 7H_2O$	0.4

DNA extraction and PCR amplification

DNA was extracted using the GeneMatrix soil DNA isolation kit (EURx 1.4, Poland), according to the manufacturer's instructions. This kit was designed specifically for the rapid isolation of pure, humic-free microbial DNA from environmental samples, and guaranteed the proper DNA isolation for the successful PCR amplification of bacteria, fungi, protozoa, algae, and others. PCR amplification was based on the method described by Agnelli et al., (2004). The primer set used in this experiment is shown in Table 3.

Table 3. Primer sequences used for PCR.

Name of primer	Primer sequence 5'-3'	Reference
27f	AGAGTTTGATC(AC)TGGCTCAG	Osborne et al. 2006
301f	GACTGGGACTTCTGGCTGGACTGGAA	Norton et al. 2002
CTO189f	CCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGRAAAG CAGGGGATCG	Kowalchuk et al. 1997
1492r	GGYTACCTTGTTACGACTT	Frank et al. 2008
302 r	TTTGATCCCCTCTGGAAAGCCTTCTTC	Norton et al. 2002

CTO654r	CYTTGTAGTTTCAAACGC	Kowalchuk et al. 1997

PCR reactions were performed in 50 µl volumes, using PCR Master-Mix (Fermentas), 1 µl of each primer (10 µM), 1 U of Taq DNA polymerase (Fermentas), 5 µl $MgCl_2$, 1 µl BSA (500 µg ml^{-1}), 1 µl 10mM dNTP's and 3 µl of DNA extracted. The PCR was performed with a BioRad MJ Mini Personal thermocycler with the following reaction conditions: 94°C for 90 s, followed by 33 cycles at 95°C for 20 s, 56°C for 30 s, 72°C for 45 s, and a final extension step at 72°C for 7 min. The PCR products, as 5 µl sub-samples, were examined by electrophoresis on 1× TAE agarose gel (1% w/v) with appropriate DNA size standards (Mass Ruler™, DNA Ladder Mix, Fermentas) to confirm the size and approximate quantity of the generated amplicons. PCR products were visualized with ethidium bromide (0.25 µg l^{-1}).
The sequencing processes were performed in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis (Institute of Biochemistry and Biophysics Warsaw, Poland). The obtained sequences were compared to the closest relatives in the NCBI Gen-Bank database by BLAST program.

Statistical analysis
Statistical analysis was performed using Statgraphics 3.0 software (STATSOFT USA). One-way ANOVA test revealed significant (p<0.05) effect of water potential on microorganisms abundance.

RESULTS AND DISCUSSION
Soil water content was expressed by a pF curve as a function of soil water tension, which provided information about the ability of soil pores to retain water at a particular water tension, which is a proxy to how tightly water was held between soil aggregates. A value of pF which is equal to 0 corresponds to the full aquatic capacity, such that all soil pores are filled with water. pF of 2.2 is typical field water capacity whereas a pF value as high as 4.2 is considered a wilting point for the plant (Stępniewska and Wolińska, 2006). Prepared pF-curves, illustrating the relations between soil water content and pF value for the investigated soil types are presented in Fig.1.
Soil samples used in the current study displayed different abilities for water retention. For example, the full water capacity (pF 0) corresponded to soil moisture between 19 and 42% v/v, and pF 3.2 to between 8 and 22 % v/v (Fig.1). Among soils investigated *Rendzina Leptosol* demonstrated the highest (14 - 42% v/v), while *Eutric Cambisol* the lowest (8 - 19% v/v) capability of retaining water (for pF 0 and 3.2 respectively).

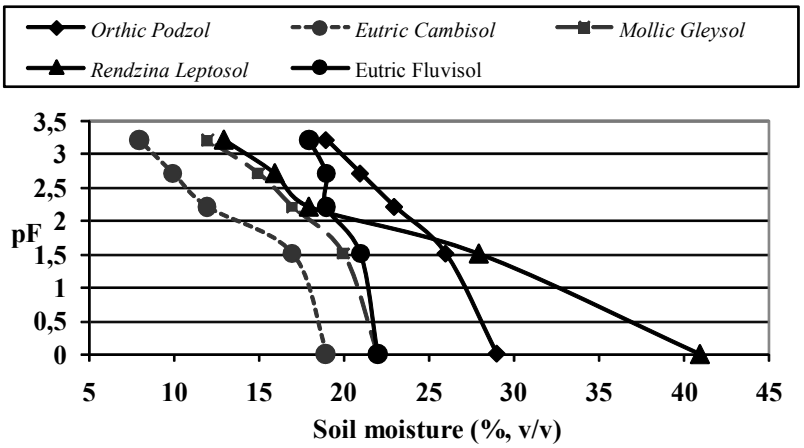

Fig1. The relationships between soil water contents and water potentials (pF curves) from 5 types of soil.

A high ability for water retention (23 – 29 % v/v) was demonstrated by *Mollic Gleysol*, and in contrast *Eutric Fluvisol* and *Orthic Podzol* showed similar abilities to retain water (14 – 22 % v/v) for both pF 0 and 3.2.

Subsequently, we examined short-term effects of soil re-moisturizing on the abundance of general, nitrifying and ammonia oxidizing bacteria examined for different soil water contents. Sensitivity of microorganism

abundance to different pF values is demonstrated in Fig.2.

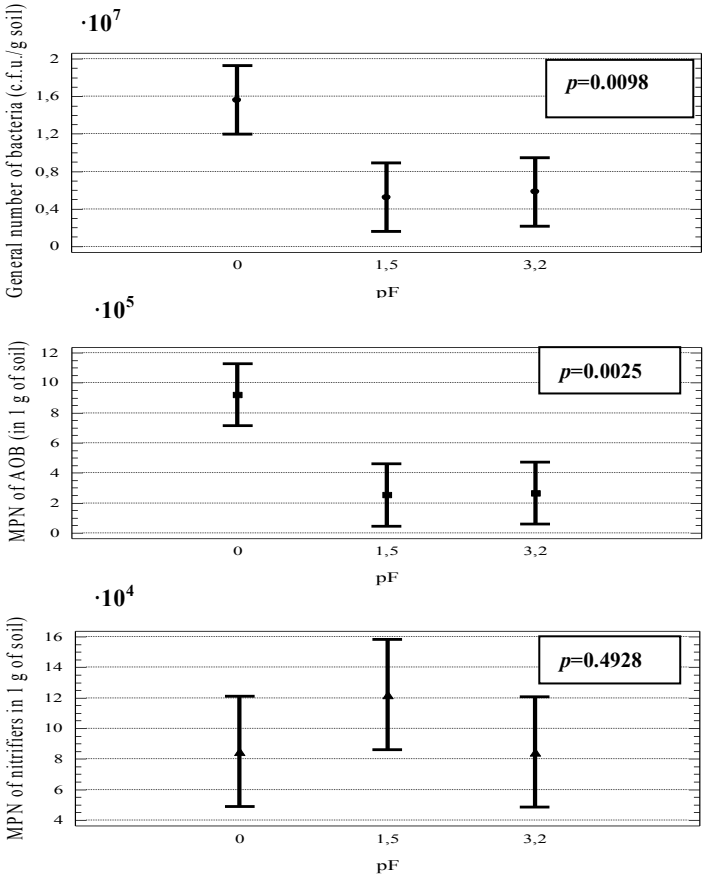

Fig 2. Total number of bacteria. MPN of AOB and MPN of nitrifying bacteria, occurring in 1 g of soil at pF 0; 1.5; 3.2. Results for the five soil types (n=45). The dots denote mean values and the bars correspond to 95% LSD intervals.

The highest number of microorganisms was noted at the saturated water conditions (pF 0), which makes sense given that water is essential for microbial survival and activity. Significant differences in soil microorganism abundance (p<0.01), are regarded as a consequence of variable pF values which were noted for both the total number of bacteria and MPN of AOB. However, MPN of nitrifying bacteria did not seem to be linked with pF (p>0.05), despite a slight decrease in the number count along with drying.

Our results are in agreement with other studies (Finlay and Esteban, 2009; Rigobelo and Nahas, 2004; Schimel et al., 2007) also showing the increase in the number of microorganisms with the increase in soil moisture. Seasonally-variable environmental factors, such as soil moisture and oxygenation may strongly influence the activity of soil microbial communities (Gleeson et al., 2008; Iovieno and Bååth, 2008; Rigobelo and Nahas, 2004).

It is known that some microbial populations have stress tolerance mechanisms more effective than others. For example, Gleeson et al., (2008) suggested that AOB, as Gram-negative bacteria, are more sensitive to rewetting stress than other soil microorganisms, which was confirmed by our results, as we consistently found higher activity and presence of AOB as a consequence of soil rewetting. Iovieno and Bååth, (2008) demonstrated that the increase in bacterial growth after rewetting was largely due to the dormant bacteria becoming active after rewetting. The activation may be a probabilistic event, similar to colony formation.

By treating individually each of the soil types (Fig. 3), it was counted that the highest abundance of the total number of bacteria occurred in *Mollic Gleysol* ($2.1\times10^7 g^{-1}$). The other representatives of investigated soils were not differing in general number of bacteria ($3 - 8\times10^6 g^{-1}$).

The second highest number of microorganism in investigated soils was AOB, reached the maximum level of its abundance ($1.1\times10^6 g^{-1}$) in *Rendzina Leptosol*.

However, a high number of AOB was also found in *Eutric Cambisol* ($7\times10^5 g^{-1}$). *Mollic Gleysol*, *Orthic Podzol* and *Eutric Fluvisol* displayed almost identical abundance of AOB ($1.8 - 2\times10^5$ g^{-1}). The number of the nitrifying bacteria populations in the investigated soils was highly

variable. The highest abundance of nitrifiers (2.0×10^5 g^{-1}) was found in *Mollic Gleysol* and *Rendzina Leptosol*. The lowest MPN of nitrifying bacteria was estimated in *Eutric Cambisol* (6×10^4 g^{-1}), but the lowest number count of these bacteria amounted to 0.5×10^4 and 1.8×10^4 g^{-1} in *Orthic Podzol* and *Eutric Fluvisol*, respectively. Our results confirmed high biological capability of the soils, despite their long term storage in the bank (at 4ºC). Ten days of pre-incubation without any limitation of water availability seemed to be sufficient for reactivation of biological life.

The microorganisms in the current study had abundance in a similar range as that published by other researchers (i.e., Dąbek-Szreniawska et al., 1996; Taok et al., 2007). Moreover, Szostak et al., (2005) highlighted that the number of nitrifying bacteria lower than AOB is acceptable, taking into account that $N\text{-}NO_3$ from nitrification process is less stable that $N\text{-}NH_4$, and thus an excess of nitrates in the soil may lead to $N\text{-}NO_3$ accumulation in the plants which can pose a health hazard for animals and people.

In order to determine the soil microorganisms groups activated as first after long period storage as a effect of soil rewetting, PCR reaction and amplified products sequencing were performed. Positive result of PCR was achieved in each of the soil types. PCR products were varying in length from 1300 - 1500 bp. Comparative analyses of the 16S rRNA sequences revealed the phylogenetic types that are constituted by the soil bacterial community. The phylogenetic division with closest database match (%) is summarized in Table 4.

Table 4. Percentage similarity based on the partial 16S rRNA gene sequences to the closest relatives in the NCBI nucleotide sequence database.

Soil sample	Closest relative in NCBI database	Accession number	Identity (%)	Phylogenetic division
Mollic Gleysol	*Pseudomonas* sp.	EU111721.1	96	*Betaproteobacteria*
	P. fluorescens	EU373377.1	96	*Betaproteobacteria*
	P. jessenii	FM209480.1	96	*Betaproteobacteria*
	P. toalaassi	FM202487.1	97	*Betaproteobacteria*
	P. putida	EU601175.1	96	*Betaproteobacteria*
	P. clemancea	AM419155.2	96	*Betaproteobacteria*
	P. teessidea	AM19154.2	96	*Betaproteobacteria*
	P. gingerii	EU14476.1	96	*Betaproteobacteria*
	P. lindanilytica	EF633256.1	97	*Betaproteobacteria*
	P. chlororaphis	EF620458.1	96	*Betaproteobacteria*
	P. moorei	FM955889.1	96	*Betaproteobacteria*
	Nitrosospira sp. *39-19*	AF042170	97	*Betaproteobacteria*
	Nitrosomonas europea	AF058692	96	*Betaproteobacteria*
Eutric Cambisol	*P. plecoglossicida*	FJ577676.1	99	*Betaproteobacteria*
	P. sp.	EU747694.1	99	*Betaproteobacteria*
	P. putida	EU258552.1	99	*Betaproteobacteria*
	P. fluorescens	FJ588702.1	99	*Betaproteobacteria*
	Nsp. multiformis 24C	AF042171	96	*Betaproteobacteria*
Rendzina Leptosol	*Delftia* sp.	EF440612.1	99	*Betaproteobacteria*
	D. tsuruhatensis	EF421404.1	99	*Betaproteobacteria*
	D. acidovorans	CP000884.1	99	*Betaproteobacteria*
	Comamonas acidovorans	AF078774	98	*Betaproteobacteria*
	D. testosteroni	M11224	97	*Betaproteobacteria*
	Nsp. tenuis NV-12	U76552	96	*Betaproteobacteria*
	Nms. europea	AF073793	96	*Betaproteobacteria*
Orthic Podzol	*Clostridium jejuense*	AY494606.1	96	*Firmicutes*
	C. *xylanovorans*	AF116920.1	96	*Firmicutes*
	C. *aminovalericum*	M23929.1	97	*Firmicutes*
	C. *citroniae*	DQ279737.1	96	*Firmicutes*
	C. *phytofermentas*	CP000885.1	96	*Firmicutes*
	Ruminococcus sp. M-1	AB125231.1	96	*Firmicutes*
	R. gauvreaui	EF529620.1	96	*Firmicutes*
	R. schinkii	X94965.1	96	*Firmicutes*
Eutric Fluvisol	*P. monteilli*	EU512943.1	99	*Betaproteobacteria*
	P. taiwanensis	EU857417.1	99	*Betaproteobacteria*
	P. mosselii	EU921228.1	99	*Betaproteobacteria*
	P. putida	EF620456.1	99	*Betaproteobacteria*
	P. plecoglossicida	FJ493170.1	99	*Betaproteobacteria*

Representatives of bacterial divisions of *Betaproteobacteria* and *Firmicutes* revealed in the study turned out to be consistent with previous literature reports. For example, Schloss and Handelsmann (2006) described that more than 92% of the 16S rRNA gene sequences were assigned to *Proteobacteria* (48.6%) and *Firmicutes* (0.8%). Similar findings were demonstrated by Dunbar et al. (2002) and Janssen (2006). In our soil types the representatives of genus *Pseudomonas* prevailed in the community with their presence at 96 - 99% identity identified in *Mollic Gleysol*, *Eutric Cambisol* and *Eutric Fluvisol*. The other soil types *Rendzina Leptosol* and *Orthic Podzol* were inhabited by microorganisms belonging to *Delftia* and *Clostridium* genus (96 - 99% identity). Furthermore, representatives of *Nitrosospira* and *Nitrosomonas* (96 - 97% identity) were discovered in *Mollic Glyesol*, *Eutric Cambisol* and *Rendzina Leptosol* samples.

CONCLUSIONS

It was demonstrated that soil rewetting with unlimited water availability resulted in reactivation of soil biological life (possibly dormant bacteria becoming active) in spite of the long period of soil storage. Variability in the abundance of microorganisms seemed related to different values of water potential (pF 0 - pF 3.2). General number of soil

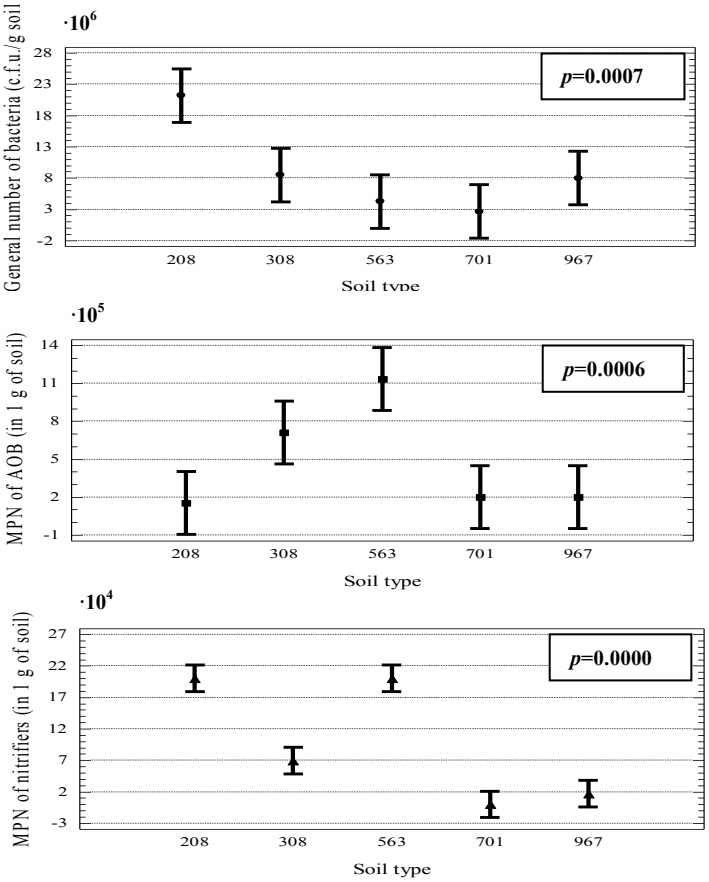

Fig 3. Total number of bacteria. MPN of AOB and MPN of nitrifying bacteria, representing individual soil types (No. 208 – *Mollic Gleysol*, No. 308 – *Eutric Cambisol*, No. 563 – *Rendzina Leptosol*, No. 701 – *Orthic Podzol*, No. 967 – *Eutric Fluvisol*). Mean values (dots) are plotted with 95% LSD intervals denoted as bars (n=45)

bacteria (p=0.0098) and MPN of AOB (p=0.0025) revealed significant negative correlations with pF, but the abundance of nitrifying bacteria was not significantly correlated ($p>0.05$) with pF, although it is possible that a small decrease in the total number count was associated with rewetting but may well have been associated with other factors such as exhaustion of available substrates.

Comparison of biodiversity in the long-term stored soils, using a combination of the PCR reaction (16S rRNA) and sequencing PCR products with identity of 96-99%, revealed that genera of *Betaproteobacteria* (*Pseudomonas*, *Nitrosospira*, *Nitrosomonas*, *Delftia*) and *Firmicutes* (*Clostridium*, *Ruminoccous*) are common bacterial groups, which quickly can

become reactivated form a dormancy state after rewetting of dry soils.

These data can be useful for future lab studies using stored soil samples and uncovered microbiological composition of dry soils after rewetting as supported by genetic analyses. Nevertheless, our understanding of the links between microbial diversity and soil environment is still largely incomplete. This could be further improved if one could identify unknown microbial communities, not cultivable yet using the current state-of-the-art molecular techniques.

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