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Microbial biodiversity in arable soils is affected by agricultural practices**

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A b s t r a c t. The aim of the study was to examine the differences in microbial community structure as a result of agricultural practices. Sixteen samples of cultivated and the same number of non-cultivated soils were selected. Gel bands were identified using the GelCompar software to create the presence-absence matrix, where each band represented a bacterial operational taxonomic unit. The data were used for principal-component analysis and additionally, the Shannon-Weaver index of general diversity, Simpson index of dominance and Simpson index of diversity were calculated. Denaturing gradient gel electrophoresis profiles clearly indicated differentiation of tested samples into two clusters: cultivated and non-cultivated soils. Greater numbers of dominant operational taxonomic units (65) in non-cultivated soils were noted compared to cultivated soils (47 operational taxonomic units). This implies that there was a reduction of dominant bacterial operational taxonomic units by nearly 30% in cultivated soils. Simpson dominance index expressing the number of species weighted by their abundance amounted to 1.22 in cultivated soils, whereas a 3-fold higher value (3.38) was observed in non-cultivated soils. Land-use practices seemed to be a important factors affected on biodiversity, because more than soil type determined the clustering into groups.

K e y w o r d s: DGGE, 16S rRNA gene, Simpson diversity, bacterial communities, arable soils

INTRODUCTION

Understanding of the main drivers influencing diversity of microbial species in soils is important as it can be related to agricultural crop yields (Lopes *et al*., 2011). The European Environmental Agency (EEA), the Commission of the European Communities (EC), European Union (EU), Biodiversity Strategy to 2020 and European project ENVASSO promote the protection strategy of microbial communities, especially in the arable soils with description of soil microbiological degradation state. It is known, that agricultural types of soil are usually biologically degraded (Wolińska *et al*., 2014), which is confirmed by the fact that arable soils are less biodiverse (Torsvik *et al*., 1998), in comparison to natural soils (typically >1000 species per g). Soil biodiversity degradation has been estimated to affect 16-40% of terrestrial areas (Girvan *et al*., 2003). Soil contains an intricate network of microbes and plants in a heterogeneous solid medium in which both chemical, physical and biological conditions vary at the molecular scale (Arias *et al*., 2005). Traditionally, soil quality has been directly related to its productivity, but more recently it has been regarded as the soil capacity to sustain biological and environmental features, and to promote plant and animal health within ecosystems (Girvan *et al*., 2003). The productivity of agricultural systems is known to depend greatly upon the functional processes of soil microbial communities (Girvan *et al*., 2003). Arias *et al*. (2005) emphasized that soil health provides an overall picture of soil functionality, whereas microbial diversity is intimately related to soil structure, and the way of land use and soil functions. A study of Kuffner *et al*. (2004) demonstrated that soil microorganisms are sensitive to anthropogenic disturbances, in particular to agricultural activities, and also

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showed that the composition of the microbiota not only can give an information about the state but also about the previous history of the soil ecosystem.

Biodiversity has been defined as the range of significantly different types of organisms and their relative abundance in an assemblage or community (Torsvik *et al*., 1998). Species diversity consists of two components: (a) species richness and (b) species evenness and/or distribution (Torsvik *et al*., 1998). Additionally, Louzpone *et al*. (2007) have distinguished diversity within each sample (α diversity) and the portioning of biological diversity among environments (β diversity). The latter could be further divided into qualitative measure, which is connected to the availability of data to compare community components, and quantitative measures, which take into account relative abundances of each organism type (Louzpone *et al*., 2007).

Community level fingerprinting methods, like denaturing gradient gel electrophoresis (DGGE) introduced by Muyzer *et al*. (1993) have distinct advantages over culture based assays, particularly in relation to describing the numerically dominant fraction of the community, a large component of which may not be conveniently cultured (Kuffner *et al*., 2004). Only a minor fraction of soil bacteria, usually estimated at 1% of the total number of cells observed by direct counting, could be cultured on laboratory artificial media (Torsvik *et al*., 1998), which is why the techniques such as DGGE can be superior for studying microbial diversity in the field and should be recommended for the microbial community studies. All prokaryotes have 16S rRNA genes whose average length is about 1500 bp (Shao-Qiang *et al*., 2012). The microbial diversity can be estimated from the number of 16S rRNA gene sequence similarity groups, *i.e*. the number of DNA bands on the DGGE gel (Shao-Qiang *et al*., 2012). Consequently, DGGE provides an estimate of the diversity within a community, based on the number of amplicons of each type, representing an operational taxonomic units (OTUs), assumed to be an equivalent to a bacterial genotype (Arias *et al*., 2005; Kuffner *et al*., 2004; Shao-Qiang *et al*., 2012). However, rare microorganisms which are ecologically relevant may not be detected using a universal approach (Kuffner *et al*., 2004). Polymerase chain reaction (PCR) in combination with DGGE can provide information about presence/absence but not about abundance of particular species due to 'qualitative nature' of PCR (Petersen and Dahllöf, 2005). Nonetheless, those techniques are necessary to understand ecological effects on biodiversity (Petersen and Dahllöf, 2005). Casamayor *et al.* (2000) reported that to be visible as a band on the gel, a species should represent at least 1% of the soil microbial community. The analysis of the soil microbiological degradation state is systematically conducted by many countries of the European Union, but this is the first such study undertaken in Poland.

Our major goal was to compare the bacterial community structure between agricultural and non-cultivated (natural) soils in order to find out the degree of microbial community degradation, caused by intensive agricultural practices. For the structural diversity determination of dominating populations PCR-DGGE technique was applied. Principal components analysis (PCA) and canonical correspondence analysis (CCA) for investigated soil bacterial communities and environmental factors effect based on DGGE profiles were also demonstrated.

MATERIALS AND METHODS

Study was performed in south-east part of Poland, in Lubelskie province (51°13′N 22°54′E) which is characterized by the great diversity of soil types and is one of the largest and most important agricultural areas in Poland where all Polish dominant soil units are present. Sixteen soil samples which were agriculturally exploited (cultivated – C) and the same number of soils not agriculturally exploited (non-cultivated – NC) were studied. Soil materials have been selected on the basis of earlier work for the typological soil recognition performed in 1991 within the framework of the Bank of Soil Samples (BSS) belonging to the Institute of Agrophysics PAS in Lublin (Bieganowski *et al*., 2013). Consequently, the fact of soils cultivation is documented since 1991 (from the foundation of BSS). Precise localization of the samples catalogued in the BSS created a possibility of the precise return to the sampling place (Gliński *et al*., 1991).

The cultivated soils were sampled during spring season (April 2014) from non ploughed places (Wolińska *et al*., 2014), according BSS locations. We consciously decided to take samples in spring season before the time when vegetation completely started and when ploughing is applied in order to avoid direct perturbations caused by tillage that affects on microorganisms destruction. The same time control samples were taken from non agriculturally cultivated and non forested sites (covering at least 1 ha area), located in close neighbouring to cultivated soils and belonging to the same soil type (*i.e*. non cultivated from years fallow lands or grasslands). In our study we focused on demonstrating those differences that resulted from long-term soil cultivation and soil 'fatigue' as those differentiation in microbial communities may have resulted from soil cultivation or the lack of cultivation for many years (20-40 years) of grasslands and/or fallow lands moved. We did not focus on the well-recognized and well described by other researchers effect of direct ploughing on soil microbial community but rather we point to those differences which do not result from direct tillage impact. Description of the fields differed by crop type and control site is presented in Table 1.

T a b l e 1. Location of agricultural soils and description of control sites (Lublin region)

Soil No.	Type of soil (FAO)	Crop type	Site	Geographic coordinates	Control sites
1	Albic Luvisol	Oat	Dęba	22º10'17.7'' 51º26'24.6''	30 year old meadow planted with fruit trees
2	Albic Luvisol	Triticale	Pryszczowa Góra	22º27'10.3'' 51º24'30.8''	20 year old woodlots with birches
3	Albic Luvisol	Wheat	Niemce	22º36'51.8'' 51º21'27.0''	50 year old meadow (mowed once a year)
4	Haplic Luvisols	Triticale	Klementowice	22º06'54.2'' 51º21'52.2''	Unmoved meadow, wasteland
5	Brunic Arenosols	Oat	Łany	22º15'19.0'' 51º23'00.9''	20 year old field-woodlots
6	Brunic Arenosols	Oat	Markuszów	22º15'55.5'' 51º23'10.9''	20 year old field-woodlots
7	Haplic Luvisol	Field prepared for seeding	Rogalin	24º04'00.3'' 50º51'15.8''	Meadow (mowed once a year)
8	Haplic Luvisols	Triticale	Sady	23º22'52.4'' 50º51'14.8''	Unmoved meadow, wasteland
9	Haplic Luvisols	Strawberries	Chrząchówek	22º07'29.9'' 51º25'50.5''	Unmoved meadow, wasteland
10	Haplic Phaezoem	Triticale	Hostynne	50º44'48.3'' 23º42'56.6''	Meadow (mowed once a year)
11	Mollic Gleysol	Colza	Pożóg Nowy	22º06'18.8'' 51º22'48.0''	30 year old pine woodlots
12	Mollic Gleysol	Wheat	Bałtów	22º01'25.5'' 51º29'15.3''	70 year old meadow (mowed once a year)
13	Eutric Fluvisol	Oat	Kośmin	21º59'10.1'' 51º33'47.7''	15 year old meadow (mowed once a year)
14	Eutric Histosol	Oat	Wólka Kątna	22º16'38.9'' 51º25'27.3''	20 year old meadow (mowed once a year)
15	Rendzina Leptosol	Celeries	Siedliszcze	23º10'58.3'' 51º12'22.3''	40 year old meadow (mowed once a year)
16	Rendzina Leptosol	Oat	Brzeziny	23º11'43.9'' 51º12'10.8''	Meadow (mowed once a year)

10 x 10 m^2 were chosen for each of the 16 different areas of the sample sites characterized by the homogeneity of the vegetation cover (Table 1). Within these squares approx. 50 random soil samples were taken from the top layer (0-20 cm) using a 2.5 cm diameter auger. Single samples were combined and homogenized into one sample in order to receive the most representative soil material for each investigated site. In this manner, 16 samples for cultivated (C) and 16 samples for control sites (NC) were obtained. Haplic Luvisols, Brunic Arenosols and Albic Luvisols are predominant soil types in Poland, occupying by 82% of the country, thus their share (nine soil samples) in the studied material was representative (Wolińska *et al*., 2014). Haplic Luvisols were represented by four soil samples (4C, 7C-9C), Albic Luvisols by three samples (1C-3C), while Brunic Arenosols by two samples (5C-6C). Mollic Gleysol and Rendzina Leptosol have also two representatives (11C-12C) and (15C-16C), respectively (Table 1). Haplic

Phaeozem (10C), Eutric Fluvisol (13C) and Eutric Histosol (14C) were represented by single soil samples. Under laboratory conditions each sample was passed through a 2.0 mm sieve, to remove large pieces of rocks and plant material and stored at 4ºC prior analysis (2-3 days).

Soil pH and redox potential (Eh) were determined in a 2:1 soil suspension in distilled water using a multifunctional potential meter pIONneer 65 (Radiometer Analytical S.A., France). Soil actual moisture was determined by a gravimetric method (24 h, 105°C), whereas total carbon (TC) using an automatic carbon analyzer TOC-V_{CSH} SSM 5000A (Shimadzu, Japan) as described by Wolińska *et al*. (2014). The concentrations of soluble phosphorus ($P\text{-}PO_4^3$), nitrate ($N\text{-}NO_3^-$) , nitrite ($N\text{-}NO_2^-$) and ammonium ($N\text{-}NH_4^+$) (were determined colorimetrically using Auto Analyser 3 System (Bran+Luebbe, Norderstedt, Germany). $P\text{-}PO_4^3$was analysed with ammonium molybdate (Banach *et al.,* 2009). $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$,were measured using, respectively, hydrazine sulphate and salicylate as a colour marker (Banach *et al*., 2009). $N\text{-}NO_2^-$ analysis were based on the latter method excluding hydrazine sulphate. Obtained results have been expressed as µg per g of fresh soil. Each analysis was performed in triplicate.

Total DNA was isolated according to the modified procedure for soil samples as described by Tomczyk-Żak *et al*. (2013). In order to reduce the presence of humic substances and other soil impurities the crude total DNA was further purified by CsCl gradient centrifugation (16 h, 70 000 rpm, 20°C; Sorvall WX Ultra ThermoScietific) as described by Sambrook *et al*. (1989). Two replicates of DNA extraction were made. Concentrations of the isolated DNA were assessed with NanoDrop spectrophotometer (ThermoScientific) after 10 times dilution.

To determine bacterial taxonomic diversity in the studied soils, a DGGE analysis of samples was performed. Dominant bacterial phylotypes were distinguished by DGGE analysis and electrophoresis performed with a D-Code Universal Mutation Detection System (BioRad Laboratories, USA). A 1-2 µl volume (roughly 5-10 ng diluted form) of each DNA was amplified by PCR mixture contained 5 µl of 10×buffer, 6.0 µl of 25 mM $MgCl_2$, 1.2 µl of 20 mg ml^{-1} BSA, 0.4 µl of 25 mM dNTP, 0.5 µl of 20 µM in each primer, 0.2 µl of 5U μl^{-1} Taq DNA polymerase (all reagents from Sigma Aldrich Co.) and 35.2 µL of PCR-grade water, in a total volume of 50 µl. The primers used were 341f with GC clamp (5'-GC- CC TAC GGG AGG CAG CAG-3') complementary to position 341–357 and 907r (CCG TCA ATT CMT TTG AGT TT) complementary to positions 926-907 *Escherichia coli* numbering (Muyzer *et al*., 1993, 1998). The PCR conditions have been previously described in Zdanowski *et al.* (2013). The samples were loaded in 6% acrylamide gels with a denaturing gradient of 35-70% (where 100% denaturant is 7M urea and 40% formamide). The same amount of DNA (40 ng) for each amplicons were loaded into the gel. The gels were run at 60 V for 15 h at 60ºC. The electrophoretic products were stained by gently agitating the gel for 30 min in 100 ml of 1×TAE containing 5 µl 1:10,000 commercial stock dilution of SYBR Gold nucleic acid stain (Invitrogen, Life Technologies, UK) in DMSO. DGGE banding patterns were visualized with UV transillumination and photographed using the Gel Doc 2000 gel documentation system (BioRad Laboratories, USA).

The bacterial community in each soil sample was compared by using DGGE fingerprinting. DGGE gel images were analyzed by Quantity One software in the GelDoc gel documentation system (BioRad Laboratories, USA). Gel bands were identified using GelCompar software to create the presence-absence matrix described by Crump and Hobbie (2005). Each band represents a bacterial OTU. The DNA bands were identified interactively, and the position and mass (intensity) of each band were determined. The data were used for principal-component analysis to evaluate differences between the DNA profiles. Qualitative PCA in which the presence but not the intensity of bands was used gave the best separation between different samples. The presence or absence of a band in each line was converted to binary matrix to access data for statistical analysis.

Dominant (well-defined) DGGE bands were excised using a scalpel blade and incubated overnight (4°C) in sterile distilled water before they were re-amplified (Chong *et al*., 2009). The PCR products were purified (Clean up, GenoPlastBiochemicals) and ligated into the pTZ57R/T (ThermoFisher Scientific) following instructions of the manufacturer. The ligation products were transformed into *Escherichia coli* DH5α using a InsTAclonePCR Cloning Kit (ThermoFisher Scientific) and cells were plated into an indicator agar which consisted of lysogeny broth (LB) medium supplemented with ampiciline (100 mg ml^{-1}), X-Gal (0.1 mM) and IPTG (0.2 mM). White colonies of transformants were replated into LB with ampiciline and after incubation the plasmid DNA was isolated (Plasmid Miniprep DNA Purification Kit, Eurx). To confirm the presence of the insert, PCR with the 341f and 907r primers was performed as described earlier. Sequencing of positive clones was carried out at by ABI3730 Genetic Analyzer (Applied Biosystems) and then, sequences were compared to those deposited in the GenBank nucleotide database. Only sequences displaying 99-100% similarity are presented here.

The relative intensity data of the DNA bands from the bacterial communities were used to carry out the following analyses and calculations. PCA of the 16S rDNA gene band patterns was performed using the Canoco v. 4.5 statistical pack (ter Braak and Šmilauer, 2002) for Windows v. software. Microbiological data for each sample were analyzed with reference to the environmental background – 'all other data'. Canonical correspondence analysis (CCA)

triplots along the two main axes of variation for the bacterial structure parameters, main environmental variables and sampled sites was also conducted.

The Shannon-Weaver diversity index, H' (Shannon and Weaver, 1963) and Simpson index of dominance (D), (Simpson, 1949) were calculated from the quantity and relative intensities of bands present in each lane according to Vivas *et al*. (2009). The digital image was analyzed, and the similarity cluster analyses based on Complete Linkage algorithm were generated by Quantity One v. 4.62 (Bio-Rad) software to express the relatedness of bacterial communities as similarity clusters. Species richness was determined as the number of bands resolved by PCR/DGGE in 1 sample lane. The similarity between the band patterns was calculated using the Dice coefficient and the clustering analysis was performed with the unweighted pair group method using arithmetic averages (UPGMA) for dendrogram construction using the STATISTICA version 10 (Stat-Soft) software package.

RESULTS

Land-use practices seemed to be a strong determinant ($p<0.05$) of soil chemical features (Table 2). As could be anticipated, agricultural soil exploitation was the reason

T a b l e 2. Chemical and biological characteristics of cultivated (C) and non-cultivated (NC) soils (±SD)

Soil No.	Moisture (%) (w/w)	pH (H_2O)	Eh (mV)	TC (%)	$N\text{-}NH_4^+$	$N\text{-}NO_3^-$	$N\text{-}NO_2^-$	$P\text{-}PO_4^{3-}$
					($\mu g\ g^{-1}$)			
1C	**8.20±0.20a**	**5.23±0.06a**	**477.4±0.40a**	**0.98±0.02a**	**0.01±0.006a**	**9.34 ±0.8a**	**0.11±0.003a**	**2.56±0.04a**
1NC	9.76±0.11b	6.27±0.005c	435.2±0.20d	1.76±0.12d	0.09±0.006d	1.68±0.014d	0.17±0.001b	1.77±0.03d
2C	**9.30±0.10a**	**4.66 ±0.02a**	**546.73±0.21a**	**1.23±0.04a**	**0.02±0.001a**	**7.37±0.05a**	**0.08±0.001a**	**1.51±0.01a**
2NC	11.16±0.11b	5.02±0.02b	528.40±0.36c	1.40±0.05b	0.04±0.014b	5.84±0.03c	0.10±0.001a	1.01±0.01c
3C	**10.22±0.03a**	**4.78±0.02a**	**535.7±0.30a**	**1.24±0.04a**	**0.01±0.001a**	**53.32±0.52a**	**0.05±0.005a**	**19.6±0.98a**
3NC	9.13±0.05b	6.22±0.09d	452.86±0.11d	1.79±0.14c	0.06±0.006d	3.58±0.09d	0.42±0.005d	1.16±0.05d
4C	**12.56±0.06a**	**6.98±0.02a**	**450.03±0.32a**	**1.96±0.05a**	**0.43±0.006a**	**18.25±0.06a**	**0.10±0.004a**	**12.9±0.04a**
4NC	13.50±0.10b	7.08±0.06a	419.20±1.11d	2.52±0.14c	0.48±0.008b	7.57±0.32d	0.53±0.003d	5.9±0.03d
5C	**6.60±0.10a**	**5.45±0.04a**	**470.20±17.75a**	**1.01±0.04a**	**0.07±0.006a**	**25.53±0.18a**	**0.12±0.001a**	**6.88±0.01a**
5NC	8.63±0.15c	5.58±0.04b	396.13±0.23d	2.06±0.19d	0.69±0.009d	10.18±0.14d	0.21±0.002d	3.52±0.09d
6C	**9.23±0.06a**	**4.78±0.006a**	**480.60±0.18a**	**0.83±0.09a**	**0.01±0.007a**	**20.26±0.07a**	**0.09±0.004a**	**4.01±0.01a**
6NC	8.63±0.15b	5.58±0.04c	396.13±0.23d	2.06±0.19d	0.69±0.009d	10.18±0.14d	0.21±0.002d	3.52±0.09b
7C	**12.13±0.15a**	**6.93±0.06a**	**403.10±3.64a**	**0.97±0.06a**	**0.05±0.001a**	**14.48±0.04a**	**0.04±0.005a**	**4.61±0.01a**
7NC	12.76±0.11b	6.99±0.03a	400.66±0.15a	3.49±0.11d	0.41±0.008d	5.41±0.14d	0.87±0.003d	3.85±0.03d
8C	**19.00±0.17a**	**5.96±0.12a**	**461.10±0.17a**	**0.96±0.11a**	**0.36±0.02a**	**17.35±0.03a**	**0.12±0.002a**	**6.81±0.02a**
8NC	20.26±0.63c	6.06±0.01a	409.23±0.25c	2.68±0.07d	2.61±0.04d	11.07±0.05d	0.24±0.02c	2.94±0.03d
9C	**5.66±0.11a**	**5.13±0.006a**	**480.73±0.93a**	**0.88±0.06a**	**0.19±0.009a**	**4.96±0.06a**	**0.14±0.001a**	**13.9±0.24a**
9NC	7.10±0.17d	5.40±0.006b	487.23±0.25b	1.42±0.11c	0.18±0.001a	1.76±0.06d	0.80±0.002d	7.52±0.02d
10C	**24.66±0.28a**	**6.61±0.05a**	**561.30±0.36a**	**1.64±0.03a**	**0.02±0.001a**	**27.43±0.08a**	**0.09±0.003a**	**1.36±0.05a**
10NC	31.03±0.23d	7.22±0.02c	529.26±0.23c	5.43±0.14d	0.02±0.002a	8.23±0.02d	0.44±0.006d	1.35±0.02a
11C	**12.96±0.28a**	**6.73±0.006a**	**556.10±0.30a**	**1.18±0.02a**	**0.41±0.04a**	**10.11±0.07a**	**0.13±0.004a**	**5.77±0.13a**
11NC	14.33±0.57c	6.76±0.01a	537.96±0.25c	3.15±0.29d	0.78±0.01c	10.06±0.09b	0.15±0.001b	1.09±0.02d
12C	**5.80±0.17a**	**4.74±0.02a**	**559.36±0.32a**	**0.91±0.05a**	**0.03±0.004a**	**21.90±0.02a**	**0.09±0.001a**	**2.04±0.03a**
12NC	10.40±0.17d	6.25±0.03d	542.90±2.95b	1.80±0.13d	4.94±0.08d	6.75±0.05d	0.10±0.001a	1.68±0.008c
13C	**5.20±0.17a**	**4.18±0.05a**	**551.30±0.30a**	**0.98±0.07a**	**0.14±0.04a**	**2.99±0.03a**	**0.09±0.001a**	**2.64±0.09a**
13NC	8.86±0.11d	5.64±0.06d	545.20±0.40a	1.23±0.08c	0.27±0.03c	2.20±0.05c	0.13±0.002b	1.33±0.008d
14C	**6.50±0.10a**	**4.85±0.03a**	**523.43±0.23a**	**2.69±0.19a**	**0.01±0.001a**	**10.22±0.12a**	**0.08±0.002a**	**3.09±0.10a**
14NC	9.30±0.20d	5.27±0.01c	519.96±0.25a	3.63±0.14d	0.02±0.002a	9.05±0.03c	0.09±0.001a	1.74±0.38d
15C	**10.86±0.11a**	**5.58±0.06a**	**503.90±0.20a**	**0.97±0.06a**	**0.05±0.01a**	**77.17±0.14a**	**0.08±0.007a**	**6.83±0.19a**
15NC	12.50±0.17c	5.76±0.01b	493.80±0.20b	1.59±0.12d	3.39±0.06d	10.12±0.07d	**0.09±0.004a**	0.60±0.007d
16C	**12.80±0.10a**	**5.58±0.11a**	**488.20±0.20a**	**1.25±0.05a**	**0.22±0.01a**	**32.98±.27a**	**0.09±0.001a**	**1.04±0.02a**
16NC	19.30±0.17d	7.39±0.02d	446.16±0.47c	5.80±0.43d	0.28±0.02b	13.82±0.5d	0.13±0.004c	1.01±0.02a

C – cultivated soils (in bold), NC – non-cultivated soils (control). The different letter indicate significant letters between C and NC ($p<0.05$).

of decrease of soil moisture, pH, TC, $N\text{-}NH_4^+$, $N\text{-}NO_2^-$. Among investigated factors Eh, $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ displayed higher values in C than in NC soils.

Generally, pH of majority of cultivatede soils was in the acidic range (4.66-5.96), whereas pH values close to neutral (6.73-6.98) were noted only in relation to four representatives of C soils (Table 2). In each experiment variant, the pH values of control soils were higher than those in cultivated soils. Cultivated soils were also characterized by lower moisture content (5.2-24.7%) than control soils (7.1-31.03%). Likewise, TC content was lowered in C variant (0.8-2.7%) in comparison to NC soils (1.4-5.8%). Dominant form of nitrogen was $N\text{-}NO_3^-$, which in C variant was remarkably higher than in NC ranged from 2.99 to 77.17, and 1.68-13.82 μg g^{-1} d.m, respectively. The second most abundant form of nitrogen in terms of quantity was $N\text{-}NH_4^+$ with the amount of 0.01-0.43 μg g^{-1} d.m. in relation to C soils and 0.02-4.94 μg g^{-1} d.m. in the NC soils. The least representative form of nitrogen was $N\text{-}NO_2^-$, at relatively low concentrations of 0.04-0.14 and 0.09-0.87 μg g^{-1} d.m. in C and NC soils, respectively.

To confirm taxonomic diversity in studied samples, a DGGE analysis was performed. This allowed for the use of CCA to determine differences, but also to assess the impact of the factors determining diversity.

Cluster analysis of DGGE profiles of C and NC soils by UPGMA demonstrated the separation of bacterial communities between cultivated and control soils bacterial communities (Fig. 1, Table 3). Generally larger numbers of OTUs (65) in NC soils were noted compared to C soils (47 OTUs). This implies that in C samples subjected to agricultural treatments the number of dominant OTUs is lower by nearly 30% with reference to non-cultivated sites. Among cultivated soils the smallest number of OTUs (17) was noted in Albic Luvisols under oat crop type (1C), whereas the largest (28 OTUs) in Haplic Luvisols under strawberries (9C) was estimated. Control sites characterized by the largest numbers of OTUs were as follows: Mollic Gleysol under 30 year old pine woods (11NC) and Haplic Luvisol under 20 years old meadow mowed once a year (14NC), where 30 OTUs were found. Rendzina Leptosol under old meadow mowed once a year (16NC) was characterized by 29 OTUs. In contrast, the lowest number of OTUs (15) was noted in Haplic Luvisol under 15 years old meadow (13NC) and in Haplic Luvisol (9NC). When comparing C and NC sites it was noticed that agriculturally exploited Albic Luvisols (1C) had the same numbers of OTUs (17) as its control (1NC). The same number of OTUs (18) also appeared in Mollic Gleysol (12C, 12NC). Among Haplic Luvisols, which were the most widely represented (4 soil samples), OTU numbers ranged from 18 to 28 in arable soils, and from 15 to 30 in controls. However, the sheer number of OTUs do not necessary imply about soil biodiversity, as arable soils were also classified by increasing diversity (Table 3), expressed as Shannon-Weaver index (H'), Simpson index of diversity (D) and dominance index (1/D). Presented comparative biodiversity along a gradient of cultivated soils clearly demonstrated that higher biodiversity (H'=1.60) was found in soils (1C, 4C, 5C, 8C, 12C, 14C) with low OTUs (17-18), whilst lower

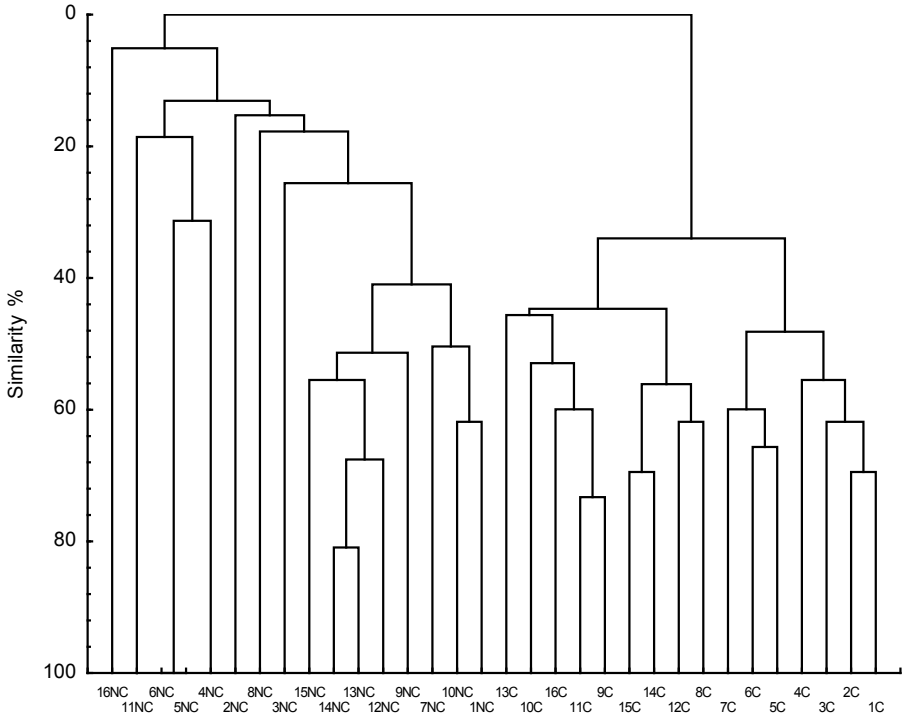

Fig. 1. Cluster analysis by UPGMA similarity of DGGE banding patterns between soil samples (NC – non-cultivated, C – cultivated). The number before N a NC indicates the soil sample number.

T a b l e 3. Comparative biodiversity along a gradient of cultivated soils

Soil type	Non-cultivated soils	H'	D	1/D	S	Cultivated soils	H'	D	1/D	S
Haplic Luvisols	9	1.47	0.049	20.41	15	9	1.34	0.045	22.39	28
Rendzina Leptosol	16	1.56	0.052	19.18	29	16	1.38	0.046	21.69	27
Eutric Fluvisol	13	1.47	0.049	20.41	15	13	1.42	0.047	21.09	26
Haplic Phaezoem	10	1.52	0.051	19.69	17	10	1.46	0.049	20.57	25
Mollic Gleysol	11	1.55	0.052	19.36	30	11	1.46	0.049	20.57	25
Brunic Arenosols	6	1.58	0.052	19.05	20	6	1.52	0.051	19.75	23
Albic Luvisols	2	1.60	0.053	18.76	26	2	1.56	0.052	19.19	21
Albic Luvisols	3	1.59	0.053	18.84	22	3	1.59	0.053	18.87	19
Haplic Luvisols	7	1.50	0.050	20.02	16	7	1.59	0.053	18.87	19
Rendzina Leptosol	15	1.58	0.052	19.05	20	15	1.59	0.053	18.87	19
Albic Luvisols	1	1.52	0.051	19.69	17	1	1.60	0.053	18.78	17
Brunic Arenosols	5	1.58	0.052	19.05	20	5	1.60	0.053	18.79	18
Eutric Histosol	14	1.50	0.050	20.02	16	14	1.60	0.053	18.78	17
Haplic Luvisols	8	1.59	0.053	18.85	26	8	1.60	0.053	18.79	18
Haplic Luvisols	4	1.55	0.052	19.36	30	4	1.60	0.053	18.79	18
Mollic Gleysol	12	1.54	0.051	19.43	18	12	1.60	0.053	18.79	18

H'– Shannon-Weaver index of general diversity, D – Simpson index of diversity, 1/D – Simpson index of dominance, and S – number of bands for DGGE profiles.

T a b l e 4. General diversity of studied soil samples based on DGGE banding patterns

Way of land use	H'	D	1/D
Non-cultivated (NC)	4.87±0.03a*	0.30±0.001a	3.38±0.05a
Cultivated (C)	5.10±0.04c	0.82±0.03d	1.22±0.4d

Different letter indicate significant letters between C and NC ($p<0.05$). Explanations as in Table 3.

biodiversity (H' from 1.34 to 1.38) have been indicated in those (9C, 16C) with the highest OTUs number (25-28). General diversity of all studied soil samples based on DGGE patterns is shown in Tables 3 and 4.

From DGGE patterns evaluated by Shannon-Weaver index we deduce a slightly higher microbial diversity in cultivated soils (H'=5.10) than in non-cultivated (H'=4.87). However, Simpson index of dominance (1/D) suggests that cultivated soils contained more specifically dominant communities (18.87-21.69) compared to non-cultivated soils where phylotype richness was lowered (18.84-20.41). Simpson index computed on the entire C and NC samples reached higher level in cultivated (0.82) than in control samples (0.30), which confirms reduced biodiversity in arable soils. This phenomenon is also highlighted by Simpson index of dominance (1/D) calculated for all DGGE tracks, describing the number of species which in arable soils amounted 1.22±0.48 whereas in controls was nearly three-fold higher reaching 3.38±0.05 (Table 4).

Dominant DGGE bands were cloned and sequenced. Representative sequences displayed 99-100% similarity with NCBI database as presented in Table 5 and Table 6, for C and NC soils, respectively. Selective DGGE bands were shared among C and NC sites, for example uncultured *Firmicutes* bacterium clones and uncultured *Acidobacteria* bacterium clones. However, in most cases

T a b l e 5. Selected species of clones obtained by PCR-DGGE in cultivated (C) soils with reference sequences in the NCBI database

Soil type (FAO)	Soil No.	Closest match from GenBank		
		Match	Sequence similarity by BLAST (%)	GenBank accession No.
Albic Luvisol	1-3C	Uncultured *Bradyrhizobium* sp. clone C.la-18	99%	JX504902.1
		Phenylobacterium sp. C16-Siri106	99%	JX500270.1
Haplic Luvisol	4P, 7-9 C	Uncultured *Xanthomonadaceae bacterium* clone GASP-MB2W2_B04	99%	EF665380.1
		Uncultured *Firmicutes bacterium* clone GASP-MB3W1_G09	99%	EF662389.1
		Arthrobacter sp. PG21	99%	KU350608.1
Brunic Arenosol	5-6C	Uncultured *Xanthomonadaceae bacterium* clone GASP-MA1S2_A03	99%	EF665874.1
		Sphingomonas sp. C0503	99%	JX096995.1
Haplic Phaeozem	10C	Uncultured *Sphingobacteriales bacterium* clone GASP-MB2W2_D09	99%	EF665405.1
Mollic Gleysol	11-12PC	Uncultured *Myxococcales bacterium* clone Plot4-2B08	100%	EU449592.1
		Mucilaginibacter sp. L356	100%	KR181805.1
		Uncultured *Acidobacteriales bacterium* clone E2006TS6.39	99%	GU983355.1
		Rhodanobacter sp. T2-YC6778	99%	GQ369046.1
Eutric Fluvisol	13C	*Rhodanobacter sp.* GR14-4	99%	KF441592.1
		Rhodanobacter sp. A2-61	99%	FJ821729.1
		Uncultured *Gammaproteobacteria* clone GC0AA3ZG11PP1	99%	JQ919685.1
		Mucilaginibacter sp. UR6-11	100%	KF900219.1
Eutric Histosol	14C	Uncultured bacterium clone WW1_a33	100%	GQ264172.1
		Uncultured bacterium clone Hswb-15	100%	GU113036.1
		Uncultured *Gammaproteobacteria* clone GC0AA4ZE09PP1	99%	JQ919693.1
Rendzina Leptosol	15-16C	Uncultured *Acidobacteria bacterium* clone AEW_08_449	100%	HQ598290.1

we noted differences between C and NC microbial communities composition. In agricultural soils (Table 5) uncultured representatives belonging to *Myxococcales*, *Mucilaginibacter* sp. L356 and *Mucilaginibacter* sp. UR6-11 were detected with 100% sequence similarity to the NCBI database. Others, displaying 99% similarity were mostly represented by uncultured clones of *Bradyrhizobium* sp., *Xanthomonadaceae*, *Gammaproteobacteria, Sphingomonas* sp., *Myxococcales, Rhodanobacter* sp., *Mucilaginibacter* sp. and *Arthrobacter* sp. With respect to control soils, 100% similarity with NCBI database was found in uncultured representatives of *Acidobacteriaceae* and *Firmicutes* bacterium clones (Table 6). Similarly, with 99% sequence similarity presence of uncultured *Hyphomicrobiaceae, Gemmatimonadetes, Caulobacterales* and *Alphaproteobacterium* bacterium clones were detec-

T a b l e 6. Selected species of clones obtained by PCR-DGGE in control (NC) soils with reference sequences in the NCBI database

Soil type (FAO)	Soil No.	Closest match from GenBank		
		Match	Sequence similarity by BLAST (%)	GenBank accession No.
Albic Luvisol	1-3NC	Uncultured *Firmicutes* bacterium clone GASP-MB2S2_C02	99%	EF665117.1
		Uncultured *Hyphomicrobiaceae* bacterium clone Elev_16S_1585	99%	EF020154.1
		Uncultured *Acidobacteri*a bacterium clone AEW_08_408	99%	HQ598261.1
Haplic Luvisol	4NC, 7-9NC	Uncultured *Acidobacteriaceae* bacterium clone CK-113	100%	KM200541.1
		Uncultured *Firmicutes* bacterium clone GASP-MA1W2_D03	100%	EF662688.1
		Uncultured *Hyphomicrobiaceae* bacterium clone Elev_16S_1697	99%	EF020212.1
Brunic Arenosol	5-6NC	Uncultured *Firmicutes* bacterium clone GASP-MB2S1_B06	100%	EF665018.1
		Uncultured *Caulobacterales* bacterium clone Plot4-G04	99%	EU449571.1
		Uncultured *Rhodoplanes sp*. partial 16S rRNA gene, clone7B_09	99%	HE861294.1
		Uncultured *Hyphomicrobiaceae* bacterium clone Amb_16S_918	99%	EF018637.1
Haplic Phaeozem	10NC	Uncultured bacterium clone FCPO743	99%	EF516120.1
		Uncultured *Hyphomicrobiaceae* bacterium clone Amb_16S_1248	99%	EF018785.1
Mollic Gleysol	11-12NC	Uncultured *Rhodoplanes sp*. clone GASP-MA1W1_B04	99%	EF662607.1
		Uncultured bacterium isolate DGGE gel band JHH-D7-1A-c35	99%	HM148942.1
Eutric Fluvisol	13NC	*Mesorhizobium sp*. AM20-87	99%	KP899163.1
		Uncultured bacterium clone FCPO696	99%	EF516451.1
Eutric Histosol	14NC	Uncultured forest soil bacterium clone DUNssu184	100%	AY913390.1
		Uncultured forest soil bacterium clone DUNssu053	99%	AY913275.1
Rendzina Leptosol	15-16NC	Uncultured *Alphaproteobacterium* clone GASP-WC1S3_B03	99%	EF074556.1
		Uncultured *Alphaproteobacterium* clone GASP-MB3W2_A04	99%	EF665890.1

ted. Furthermore, in NC soils uncultured *Rhodoplanes* sp., *Mesorhizobium* sp. and forest soil bacterium clones were also identified.

Effect of agricultural practices on soil biodiversity reduction was also determined by performing the principal component analysis. DGGE profiles revealed clear differences in microbial diversity of the soil samples (Fig. 2). Cultivated soil samples demonstrated increased affinity to each other by forming a clear cluster, in contrast to controls where samples (3NC, 4NC, 6NC, 11NC) differed from samples in other positions. Principal component analysis also pointed to a greater similarity of C soils within the group with less resemblance to NC sites.

Chemical factors effect on investigated soil types (C and NC) based on DGGE patterns are presented in Fig. 3. The correspondence analysis has shown that the overall variability index (Total Inertia) makes it possible to apply the linear fit. The PCA analysis revealed a significant variability in the samples. This highlights the need for conducting further analyses in the CCA system relative to the background. Our results suggest that Eh and $N\text{-}NO_3^-$ are the critical factors controlling microbial communities in arable soils, particularly with regards to Haplic Luvisols (4C, 9C), Mollic Gleysols (11C, 12C) and Rendzina Leptosol (15C). Microbial community from control sites grouped in the right bottom quadrant (1NC, 4NC, 7NC, 8NC, 10NC, 14NC, 16NC) depended on soil moisture and TC content. On the other hand, biogenic forms of phosphorus ($P\text{-}PO_4^{3}$), nitrogen ($N\text{-}NO_2^-$, $N\text{-}NH_4^+$) and pH, affecting microbial community from non-cultivated soils, clustered in the top right quadrant (3NC, 11NC, 12NC). The Monte Carlo Permutation test revealed that moisture, TC and pH were the three important factors responsible for effects at a statistically significant level.

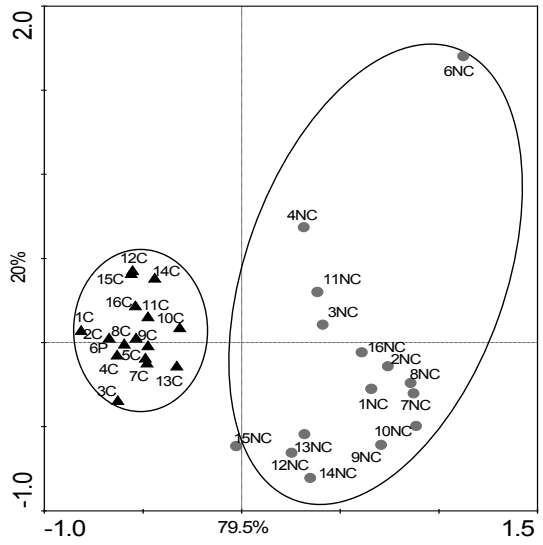

Fig. 2. Principal components analysis (PCA) of DGGE band patterns. Numbers beside the symbols indicate the soil samples number, circle – non-cultivated soils (NC), triangles – cultivated soils (C).

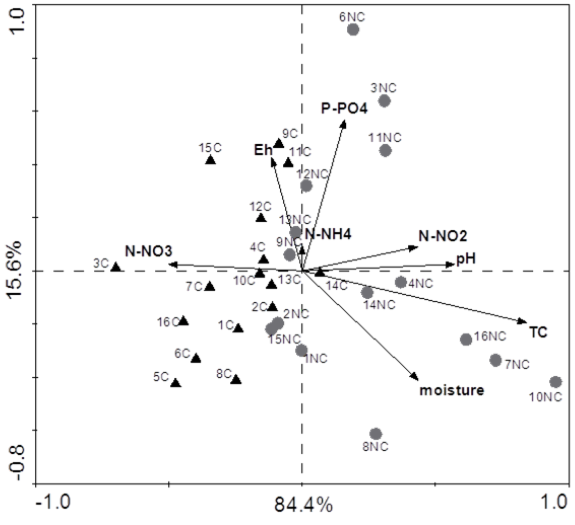

Fig. 3. Canonical correspondence analysis (CCA) for the positions and chemical factors based on DGGE profiles. Numbers beside the symbols indicate the soil samples number, circle – non-cultivated soils (NC), triangles – cultivated soils (C).

DISCUSSION

The fact that soil agricultural treatments strongly modify soil chemical parameters was reported by Girvan *et al*. (2003), Lopes *et al.* (2011) and Wolińska *et al.* (2014). Also decline of pH toward acidic conditions as an effect of agricultural practices and depletion of TC in arable soils was earlier indicated (Bossio *et al*., 2005; Wolińska *et al*., 2014). These observations are consistent with results presented in the current study, where decrease of pH and TC content was noted in C soils. It was stated that vegetation type and carbon concentration may influence microbial community composition at local scales, whereas soil pH is a better predictor of a community structure at the continental scale (Fierer and Jackson, 2006). Observed increase in Eh in C soils was linked to the fact that agricultural practices, such as ploughing, contribute favourably to oxygen enhancement in the soil surface, whereas growth of $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ may be caused by systematically fertilization practices in arable soils. Domination of nitrate nitrogen in agricultural soils especially during the spring season can be explained by agricultural practices such as fertilization, and high soil temperature that stimulate aerobic N transformation, resulting in the nitrification of of $N\text{-}NH_4^+$ (Campos, 2010). Furthermore, one extremely important observation seems to be the marked reduction of $N\text{-}NO_2^-$ concentration in all cultivated soil which suggests that microbiological processes associated with nitrogen compounds in cultivated soils may prevent the accumulation of $N\text{-}NO_2^-$. Similarly, a decrease in $N\text{-}NH_4^+$ noted in cultivated soils might suggest on intensive nitrification processes in C soils. It should be mentioned that we collected the soil material prior to

fertilizer application. Thus, noted concentration range for $N–NH_4^+$ resulted from its pool that remained in the soil after winter time. Our data are consistent with the findings of Avrahami *et al.* (2003) who observed that even after the direct fertilization of soil with $N\text{-}NH_4^+$ a decrease of ammonium and increase in nitrate concentrations occurred. This phenomenon was favoured by intermediate air temperatures, *i.e*. 15-25°C, whereas during soil sampling in spring 2014 the air temperature was in the range of 20-22°C.

An impact of land management practices on bacterial diversity structure still remains unrecognised. It has been suggested that agriculture creates highly selective and homogeneous environments that reduce bacterial diversity, particularly *Rhizobium* populations (Palmer and Young, 2000). Some studies were performed in order to report on the differences in microbial diversity between soils under conventional and organic farming systems (Bossio *et al.,* 2005; Kuffner *et al*., 2004; Lopes *et al.,* 2011). In that context, the present study is the first one that compared the bacterial community in cultivated and non-cultivated Polish soils, represented by seven types and sixteen soil units. We found that, differentiation between cultivated and non-cultivated soils on the level of chemical features is clearly a consequence of different soil management practices and this was also the reason for the diversification in the microbial community structure. In our study land-use management seemed to be a major determinant of the bacterial communities, because more than the soil type it determined the clustering into groups (Fig. 1) and showed clear differences in microbial composition between C and NC sites (Tables 5 and 6). This dependency is well shown based on uncultured *Hyphomicrobiaceae* bacterium clones whose presence (99-100% similarity) has been demonstrated in Albic Luvisols, Haplic Luvisols, Brunic Arenosols and Haplic Phaeozem (Table 6). Also, representatives of *Xanthomonadaceae* bacterium clones were common across Albic Luvisols and Brunic Arenosols (Table 5). In contrast, *Gammaproteobacteria* were noted both in Eutric Histosol and Eutric Fluvisols (Table 5). Similarly, the presence of *Rhodanobacter* sp. was stated both in Mollic Gleysol and Eutric Fluvisol, whereas *Mucilinibacter* sp. were common across Eutric Histosol and Mollic Gleysol (Table 5). Bossio *et al*. (2005) confirmed that different management practices affected both microbial community composition and function. In contrast, Girvan *et al*. (2003) suggested that it is likely that the total bacterial community compositions have been determined primarily by the underlying soil chemistry and structure rather than by the different management practices or cropping regimens at these sites. Additionally, soil has been shown to have an immense capacity for diversity and therefore a large buffering capacity before the results of management practices will likely affect the dominant members of the community (Girvan *et al*., 2003). In any case, it should be emphasized that the longer-term impacts of management practices may be much more significant, than what was shown in the current study.

The analysis of DGGE profiles based on the diversity of 16S rRNA bacterial gene clearly demonstrated two separate clusters for C and NC soils. These findings are also confirmed by Shannon-Weaver index of geneal diversity (H'), Simpson index of diversity (D) and Simpson index of dominance (1/D). This diversity indices clearly point to biodiversity loss in a systematical and long-term exploited arable soils, regardless of the soil type. Specifically, this fact has been the most strongly highlighted by Simpson index of dominance, where its value pointed for NC sites threefold exceeding the 1/D level estimated in C soils by a factor of 3. Lopes *et al*. (2011) reported Shannon-Weaver index for cultivated paddy Portugal soils under conventional system on the level of 1.26, meanwhile in our study H' achieved values of 1.34-1.60 and 1.47-1.560, for cultiated and control soils, respectively. Shao-Qiang *et al*. (2012) indicated that in Chinese alluvial soil under rice-wheat cropping system the highest genetic biodiversity (H'=3.061) characterized fertilized soils, while the poorest biodiversity (H'=2.692) was found in control (non fertilized) soils. These results suggest that investigated soils from the Lublin region have higher biodiversity than paddy soils from Portugal but lower than alluvial China soils.

PCA analysis also indicated a clustering among the C soils that were associated with soil cultivation and NC soils under any cultivation system for 15-30 years. This might indirectly confirm the impact of agricultural management on the bacterial communities in the soils. Apart from human agricultural management, soil microbial community is dependent on chemical features of the site. Our study demonstrated that moisture, TC and pH affected on microbial community in control soils, while Eh and $N\text{-}NO_3^-$ influenced microorganisms in cultivated soils. It is worth mentioning that moisture in control sites was by 20-30% higher than in agricultural soils. TC in NC variant reached on average 70% higher level than in C soils, whereas pH in controls was close to neutral in contrast to acidic conditions noted in arable soils. All these factors are important for microbial activities. Similarly, changes in soil moisture status is known to affect the magnitude of biological activities, because many soil microorganisms are known to be intolerant of low moisture content (Wolińska *et al*., 2015). The next important, environmental factor, expressing the tendency of an environment to receive or to supply electrons in solution is The well-oxygenated soils are characterized by high values of Eh (600-800 mV), in quite well-oxygenated soils Eh ~ 500-600 mV, whereas in anaerobic conditions a decline in Eh below 300 mV or even to lower values was observed (Pett-Ridge and Firestone, 2005; Wolińska *et al.,* 2014). It is well known, that Eh plays a crucial role in regulating microbial activity as well as community structure (Pett-Ridge and Firestone, 2005). In terms of the soils

investigated in the current study, Eh strongly influenced on microbial community in C soils (Fig. 3), where it amounted from 403 to 561 mV, whereas reached lower values (396-545 mV) in NC variant (Table 2). Also amounts of NO_3-N and PO_4-P were higher in agricultural soils, which resulted from systematically fertilization of C sites (at least from 25 years, since 1991) and affected on microbial community in arable soils. In our study, limitation of water, lower content of TC, higher level of Eh and NO_3-N concentrations and acidic pH noted in arable soils seemed to be the important factors responsible for the clear differences between the C and NC soil samples, with regard to dominant OTUs. This knowledge can be instructive for the optimal land-use management practices and enhance sustainable agriculture.

CONCLUSIONS

1. The biodiversity of microbial communities is different in the areas under cultivation than in non-cultivated soils.

2. Human agricultural activity and soil chemical parameters are important, selective factors for bacterial selection in arable soils.

3. Larger numbers of operational taxonomic units were found in control soils compared to agricultural soils, which indicates an almost 30% reduction in dominant bacterial operational taxonomic units. These is additionally confirmed by Simpson dominance index of 1.22, expressing the 'abundance weighted true diversity', whereas in the controls this index reached 3.38 which is by almost a factor of 3 higher.

4. Agricultural soil usage is the factor crucial for biodiversity structure

5. The most important key chemical factors associated with microbial community compositions turn out to be soil moisture, pH, TC, Eh N-NO_3^- and P-PO_4^{3-}, but, their impact depended on the land-use management practices.

6. In cultivated soils the most critical for microbial community factors are Eh and NO_3-N, whereas moisture, pH, TC and P-PO_4^{3-}seemed important in non-cultivated sites.

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