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MEANS OF MOLECULAR NUCLEIC ACIDS ANALYSIS IN SOIL INVESTIGATIONS

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LIST OF SYMBOLS USED

ARDRA – amplified ribosomal DNA restriction analysis,
ARISA – automated ribosomal intergenic spacer analysis,
bp – base pair,
BSA – bovine serum albumin,
Chelex-100 – a chelating material from Bio-Rad used to purify other compounds via ion exchange,
CLPP – community level physiological profile,
CTAB – hexadecyltrimethylammonium bromide,
cDNA – complementary DNA,
DGGE – denaturing gradient gel electrophoresis,
DNA – deoxyribonucleic acid,
DNase – deoxirybonuclease,
dNTP – deoxyribonucleoside triphosphates,
dsDNA – double stranded DNA,
eDNA – extracellular DNA,
EDTA – ethylenediaminetetraacetic acid,
Etc. – et cetera (Latin), and so forth,
FAM - phosphoramidite fluorochrome 5 carboxyfluorescein,
FISH - fluorescent *in situ* hybridization,
GC – gas chromatography,
G+C – guanine + cytosine,
h – hour,
iDNA – intracellular DNA,
i.e. – id est (Latin), that is,
IGS – intergenic spacer,
ITS – internal transcribed spacer,
kb – kilobase,
mM – "milliMolarity", millimoles,
mRNA – matrix ribonucleic acid,
PCR – polymerase chain reaction,
PEG – polyethylene glycol,
pF – water potential,
PLFAs – phospholipid fatty acids,
pmol – picomoles,

PVPP – polyvinylpolypyrrolidone,
rDNA – ribosomal DNA,
RFLP – restriction length fragment polymorphism,
RISA – ribosomal intergenic spacer analysis RNA,
RNA – ribonucleic acid,
RNase – ribonuclease,
rRNA- ribosomal RNA,
RSGP – reverse sample genome probing,
RT-PCR – reverse transcriptase polymerase chain reaction,
qPCR – quantitative polymerase chain reaction,
SDS – the detergent, sodium dodecyl sulfate,
SSCP – single stand conformation polymorphism,
TAE – Tris-acetate-EDTA buffer,
TBE – Tris-borate-EDTA buffer,
TE – Tris EDTA Buffer,
TET – 4.7.2V.7V-tetrachloro-6-carboxyfluorescein,
TGGE – thermal gradient gel electrophoresis,
T-RFLP – terminal restriction fragment length polymorphism,
tRNA – transfer RNA,
vs. – versus,
wt /w t% - the number of grams of solute in 100 grams of solution.

1. INTRODUCTION

One of the most intensively discussed issues in the recent decades is the loss of biological diversity. Soil environment is, however, the major reservoir of microbial genetic diversity and thus should be particularly protected.

The United Nations proclaimed 2010 to be the International Year of Biodiversity, and people all over the world are working to safeguard this irreplaceable natural wealth and reduce biodiversity loss.

Microbial diversity represents complexity and variability at different levels of biological organization. It encompasses genetic variability within taxons (species), number (richness), relative abundance (evenness) and functional groups (guilds) in communities.

The breakthrough in biology was the discovery of DNA, which is a carrier of biological information and the best characteristics of every organism. The total soil DNA includes both intracellular as extracellular DNA forms, with the latter originating from the former by active or passive extrusion mechanisms and by cell lysis.

However, the major part of soil DNA derives from plants (replication of DNA during cell division or plant development). Plant DNA could also enter into the soil through pollen release and the plant material addition to the soil. Another source of DNA are soil fauna and fungi, but most of all DNA is released by lysis process mentioned above (i.e. after bacterial infection by phages, or as a consequence of cell death).

Past environmental studies were focused on studying particular aspects of the soil, like: soil respiration activity, fertility or cycling of elements, and delivered rather little information which could be helpful in obtaining full insight into soil life aspects.

Recent extensive studies thanks to the molecular techniques development are concentrated on soils microbial world i.e. soil biodiversity analysis, contribution of nucleic acids to nutrient cycling in the environment, or interrelations between microorganisms and soil structure function.

The use of molecular techniques has improved the determination of the composition of soil microflora as most of them give fingerprints that allow to study special and temporal variations in the microorganisms community. Thus soil DNA analysis are considered to be important and precise tool towards a better recognition of soil microbial functionality and relationships among them. Anywise, the nature of subsequent analyses requires a special preparation of soil sam-

ples and extracts pure enough to give a reliable results, what might be troublesome in practice.

The aim of presented review is to compile the current knowledge about the most popular molecular methods used in the soil investigations. By presenting advantages and disadvantages of some molecular tests we would like this book will be helpful in:

1. choosing an optimal method for DNA isolation,
2. selection of the technique leading to microbial identification, and
3. cataloging the biodiversity of soil bacteria.

2. SOIL AS A RESERVOIR OF BIODIVERISTY

Soil is a place for living for some of the most fascinating organisms on Earth. Ecologists describing microbial biogeography typically invoke Beijerinck from century ago: “Everything is everywhere, the environment selects” (Fierer and Jackson 2006).

Practically, very often we do not realize that below our feet existing an immensely complex web of life, that includes the smallest bacteria, fungi and burrowing mammals. The diversity in soils is several orders of magnitude higher than that above ground and it seems to be the last frontier for biodiversity on Earth (Black *et al.* 2003).

Soils make the Earth habitable. It does this most of all by (Rimmer 1998):

- being the interface (Fig. 1) between the atmosphere (air), the geosphere (rock), the hydrosphere (water) and the biosphere (organisms),

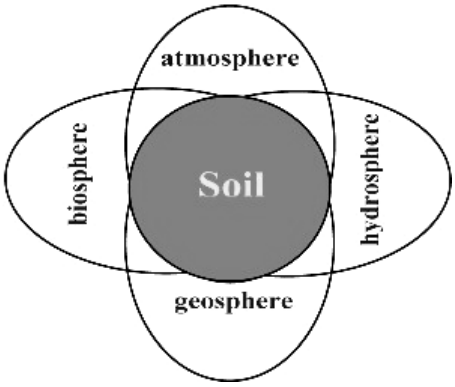

Fig. 1. Soil as the interface linking the four spheres: atmosphere, hydrosphere, geosphere and biosphere (according to Rimmer 1998)

- housing the important life supporting cycles: the energy cycle, the hydrological cycle, the carbon cycle, the nitrogen cycle – in effect a self regulating biological factory.

A peculiarity of soil as a biological system (Fig. 2) is its structure, heterogeneity and discontinuity, affecting its properties as a place for the activities of organisms living in discrete microhabitats, called “hot spots”, representing a little ratio (generally lower than 5%) of the overall available space (Nannipieri *et al*. 2003, Agnelli *et al*. 2004, Sey *et al*. 2008, Wolińska and Stępniewska 2011).

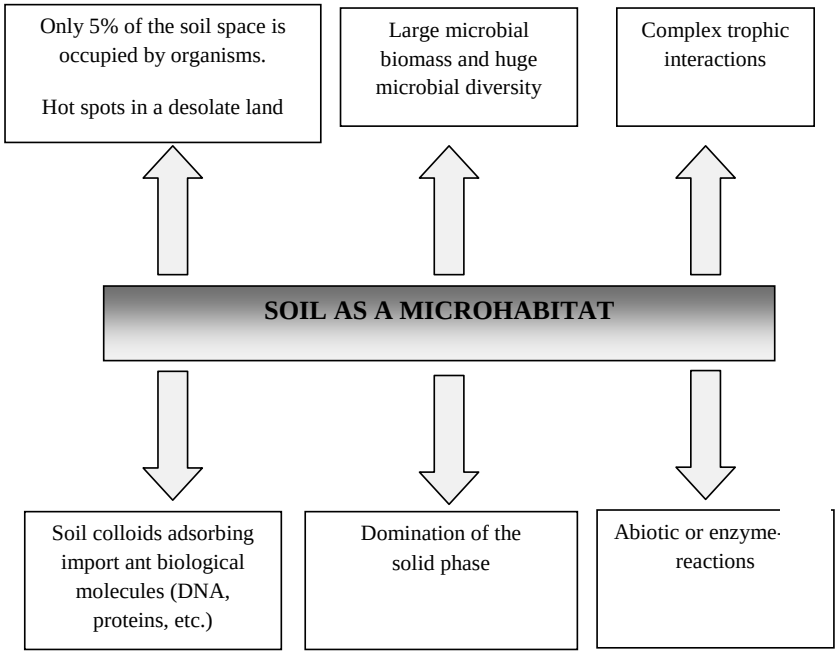

Fig. 2. Schematic representation of the main characteristics of soil as a microhabitat (according to Nannipieri *et al*. 2003)

Daniel (2005) suggested that microhabitats for soil microorganisms include the surface of the soil aggregates, and the complex pore spaces between and inside the aggregates. Young and Ritz (2000) introduced the term of “habitable pore space”, suggesting that there is a relation between size of organisms and the zones of soils they are physically able to inhabit. Analysis of the spatial distribution of

bacteria at soil micro-aggregates indicated that they include 80% of soil bacteria (Young and Ritz 2000, Torsvik and Øvreas 2002).

Interestingly a study by Torsvik and Øvreas (2002) indicated that soil microbes are more diverse in microaggregates than macroaggregates, and that there is specificity in soil microbial community with regard to the soil particles properties. For example, *Acidobacterium* were associated with small particles (silt and clay), whereas α-*Proteobacteria* were associated with large particles (sand). Smit *et al.* (2001) suggested that nutrient availability determined variation of bacteria in the soil particles as increased nutrient availability resulted in α- and γ-*Proteobacteria*, with *r*-selection, which is characteristic for bacteria with potentially high growth rates. However, soils, which were not rich in nutrients and high in recalcitrant substrates, represented by *Acidobacterium*, being indicative of *k*-selection, the characteristic for bacteria with lower growth potential but higher capability to compete substrates.

All microorganisms are aquatic and they live free or attached to surfaces, in water films surrounding solid particles, and inside aggregates (Nannipieri *et al.* 2003, Dexter 2004). In each gram of soil, billions of microorganisms are found, whose activities can greatly affect biogeochemical cycles (Schloss and Handelsman 2006, Włodarczyk and Brzezińska 2007). An average content of soil inhabitants estimated in the surface layer of the cultivated soils is presented in Table 1.

Table 1. An average content of microorganisms in the cultivated soil to the level of 25 cm (according to Libudzisz *et al.* 2007)

Microorganisms	Number of microorganisms at 1g of soil
General number of bacteria, contained:	$5 \cdot 10^7$
Actinomycetes	$2 \cdot 10^6$
Ammonia oxidizing bacteria	$4 \cdot 10^6$
Denitrifying bacteria	$1 \cdot 10^5$
Aerobic bacteria	$9 \cdot 10^6$
Anaerobic bacteria	$2 \cdot 10^6$
Fungi	$1 \cdot 10^5$

Torsvik *et al.* (1990) indicated that there are 4000 different bacterial "genomic units" only in 1 g of soil. It has also been estimated that about 5000 bacterial species have been described so far (Pace 1997). However, only 1% of the soil bac-

terial population can be cultured by standard laboratory practices, but it is not known if this 1% is representative of the bacterial population (Kirk *et al.* 2004, Fierer *et al.* 2007).

We should remember, that all organisms in the biosphere depend on microbial activity (Pace 1997). It is well known that soil microorganisms which colonize the rhizosphere assist plants in the uptake of several vital nutrients from soil, such as phosphorous, potassium and nitrogen. Soil microbes can also exert considerable influence upon the status of a plant's health.

This is the reason why there has been a surge of interest in soils as reservoir of biodiversity in the last several years. Finding relations between diversity and function in soil ecosystem has become one of most important challenges in soil biology and microbiology. However, before we can address how changes in microbial community structure influences ecosystem functions, there is a need for reliable and accurate mechanisms of soil microorganisms studying.

The last 15 years have brought many technological advances in community profiling and cultivation in dependent approaches to studying of soil microorganisms. Many researchers argue that it is now possible to explore the ''black box'' of soil microbial communities. Soil DNA analysis might become the next step towards better understanding and recognition of interrelations existing in the biological soil system.

3. DNA IN THE SOIL ENVIRONMENT

Nucleic acids are ubiquitous compounds in soil. Most of the information about soil microorganisms' ecology and diversity is lodged in the genetic material occurring in this complex environment.

The discovery of the DNA molecule was the breakthrough in biology. The differences among the sequences in the DNA chains compose the genetic diversity, which may appear as a biological diversity in structure, and organization.

Once released into the environment eDNA may:

- persist by binding to soil minerals and humic substances (Crecchio and Stotzky 1998),
- be degraded by microbial DNases and used as a nutrient for plant and microbial growth (Ceccherini *et al.* 2003), and/or
- be incorporated into a bacterial genome as a possible source of genetic diversity (Levy-Booth *et al.* 2007).

DNA extracts are generally obtained to be analyzed with the use of molecular techniques. These, according to their sensitivity, can detect species, families, or even higher taxonomic groups (Nannipieri *et al*. 2003), could be used to determine the relations between genetic diversity and community structure (Lee *et al.* 1996), be applied to investigate the impact of the tillage treatments (Six *et al.* 2004), soil contamination (Malik *et al*. 2008), and introduction of genetically modified organisms into the environment (Lukow *et al*. 2000).

The general content of DNA varies in relation to the soil types, soil conditions, numbers of microorganisms, type of cultivation, climate etc. Study performed by Wolińska (2009) demonstrated DNA content in the different soil types, taken form Bank of Polish Soil Samples belonging to Institute of Agrophysics PAS in Lublin (Tab. 2).

Table 2. DNA content at the surface layer (0-30 cm) in the different soil types (after 10 days incubation in 20ºC at flooded conditions) at pF 0 and pF 3.2 (according to Wolińska 2009)

Soil type (FAO)	DNA concentration (μg g^{-1})	
	pF 0	pF 3.2
Orthic Podzol	57.3	35.8
Eutric Cambisol	6.1	5.6
Mollic Gleysol	11.5	10.7
Eutric Fluvisol	49.7	43.5
Rendzina Leptosol	52	42
Eutric Histosol	98.5	85.2
Haplic Phaezoem	190.8	179.4

Generally higher DNA concentration at full water capacity conditions (pF 0) was noted (Fig. 3), however the differences were not statistically significant (p>0.05). These observations remained in agreement with work of Schimel *et al.* (2007) and Finlay and Esteban (2009).

Fritze *et al.* (2000) and Agnelli *et al.* (2004) indicated that DNA content in soil environment usually ranged between 0.03-200 μg g^{-1}.

Current approach aims the general insight into the cycling of soil eDNA, its ecological relevance as a source of genetic information and nutrients for microor-

ganisms, the role of DNA in bacterial biofilm formation and the contribution of the genetic information to better evaluation of the microbial communities composition in soils (Pietramellara *et al*. 2009). Each DNA cycle begins with the entry and potential persistence in soil.

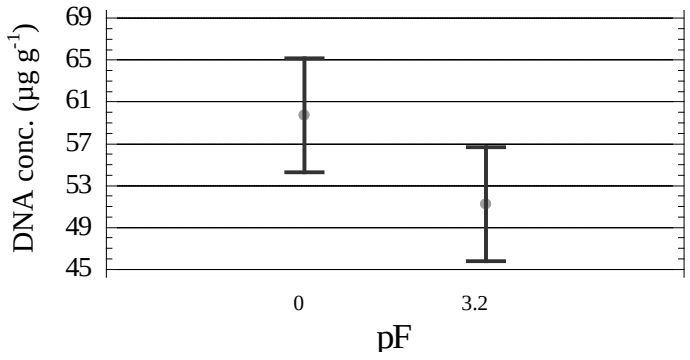

Fig. 3. DNA concentration (µg g^{-1}) at surface layers of investigated soil, at water potential value of: pF 0 and pF 3.2 (n = 21, $p > 0.05$), according to Wolińska (2009)

3.1. DNA entry into the soil

The literature data demonstrated that a major part of soil DNA derives from plants (replication of DNA during cell division or plant development). Plant DNA could also enter into the soil through pollen release and the plant material addition to the soil (Stokstad 2003, Pote *et al.* 2007).

However, according to Levy-Booth *et al.* (2007) most of soil DNA is released by lysis process (i.e. after bacterial infection by phages, or as a consequence of cell death). Numerous genera of bacteria release their DNA during biofilm formation by growth in liquid media (Lorenz and Wackernagel 1994).

Finally, during the decomposition of dead cells and tissues, particular environmental conditions, such as rapid dissection, low temperatures, high salt concentrations or low pH values, can affect the activity of the endoenzymes, increasing the persistence of both cells or tissues as well as the DNA (Pietramellara *et al*. 2009).

DNA released into the soil environment rarely is present as a pure molecule. Generally, it is present as a constitutional organic component either enters into other biological cycles or persists in soil as a result of DNA-soil particles interrelations (Tamayo *et al*. 1999).

3.2. DNA adsorption on soil molecules

DNA is negatively-charged, thus it can absorb easily to net positively-charged surfaces, but in the case of net negatively-charged surfaces DNA molecules require cations to mediate adsorption.

DNA can be incorporated in surfaces between Al-hydroxide layers of clay minerals by simple ion-exchange reactions (Crecchio *et al*. 2005) or it can be absorbed by electrostatic bridges with water as a result of hydration of charge-compensating cations (Lorenz and Wackernagel 1994). Adsorption of DNA to various purified minerals has been found to increase with increasing monovalent (Na^{+}, K^{+}, NH^{4+}) and divalent (Mg^{2+}, Ca^{2+}) cation concentrations (Paget *et al*, 1992, Lorenz and Wackernagel 1994). What is more, it was demonstrated that divalent cations are substantially more efficient DNA adsorption mediators than monovalent cations (Lorenz and Wackernagel 1994).

When adsorption of DNA to the minerals takes place, it is a rapid process. The time required for half-maximum adsorption to purified sand or clay minerals and to sediment material (groundwater aquifer) lies between <1 and 15 minutes at 23°C (Khanna and Stotzky 1992, Lorenz and Wackernagel 1992).

Complexes of DNA with soil components, such as montmorillonite, clay, humic substances and Al or Fe minerals are believed to be the primary forms of DNA protection against DNases (Crecchio and Stotzky 1998).

Binding of DNA on soil minerals and organic substances depends on both, properties of particular soil mineral and DNA characteristics. It is generally believed that smaller DNA fragments adsorbed within pores would be protected from degradation by nuclease more effectively than fragments adsorbed to surface sites.

Ogram *et al.* (1994) hypothesized that a size exclusion effect may occur only above a certain (as-yet-undetermined) DNA length, below which sorption would be limited by decreasing numbers of sorptive moieties of DNA and by available adsorption sites within the pore. The adsorption efficiency may also be related to differences in diffusion rates between the larger and smaller polymers (Williams and Williams 1973). The higher diffusion rate of the smaller polymer would lead to faster adsorption kinetics, possibly resulting in the smaller DNA fragment outcompeting the larger for adsorptive sites (Ogram *et al.* 1994).

On the other hand, Pietramellara *et al*. (2009) indicated, that the higher molecular mass DNA can interact with larger number of binding sites on the external surfaces of clay minerals than the lower molecular mass. The number of binding sites and binding mechanisms are important in determining a soil particle-DNA binding

capacity. A study by Blum *et al.* (1997) found that in a loamy sand DNA adsorption proceeds most easily and reaching its maximum after one hour upon entry.

Some authors suggested that the capacity of sand to bind DNA is at least three orders of magnitude lower than that of clay (Khanna and Stotzky 1992, Paget *et al.* 1992, Blum et al. 1997). Clay minerals have a higher surface area as a result of the micelle structure of the particles and the higher net negative charge (Stotzky 1986).

However, it was assumed that clay minerals differ in DNA adsorption rates. For instance, Khanna and Stotzky (1992) and Pietramellara *et al.* (2009) demonstrated that montmorillonite 2:1 (Si:Al) adsorbed DNA more efficiently than kaolinite 1:1 (Si:Al).

It is also known, that in a sand-clay microcosm 60% of the adsorbed DNA is located on bentonite clay, which made up only 0.6% of the mineral material (Lorenz and Wackernagel 1992). Similarly, Ogram *et al.* (1998) noted the greater extent of DNA adsorption in a soil containing montmorillonite because of it high binding capacity in comparison to acid-washed sand (no clay).

Silt, a class of intermediate soil particles has a DNA binding capacity between that of sand and clay (Levy-Booth *et al.* 2007). A small number of DNA molecules can also be adsorbed on silica, on which few inorganic anions adsorb because of the strong negative charges (Saeki and Matsumoto 1994).

Finally, as shown by Khanna and Stotzky (1992) and Paget *et al.* (1992) DNA molecules may also be bound directly to soil organic matter. Binding sites of humic substances are formed when phenolic hydroxyl groups within these compounds are ionized.

Analogically, acidic functional groups of humic substances which arise mainly from carboxylic acid, may also occur as the binding sites (Stevenson 1994).

3.3. DNA persistence and degradation

Several studies performed with components of sand (quartz, feldspar), and natural material (soils and groundwater aquifer material) indicated that DNA adsorbed on these materials is considerably less susceptible to enzymatic hydrolysis than dissolved form (Lorenz and Wackernagel 1987, Romanowski *et al.* 1993). However, the mechanism of DNA protection against nucleolytic degradation is still not fully understood.

It has been assumed that DNase finds only limited access to the adsorbed DNA molecules (Lorenz and Wackernagel 1987, Khanna and Stotzky 1992, Paget *et al.* 1992).

Alternatively, since DNase adsorbs itself to sand and clay, the enzyme may be inhibited or inactivated in a similar way to that observed with several other clay-adsorbed compounds (Khanna and Stotzky 1992, Lorenz and Wackernagel 1994).

The reduction of enzymatic activity may be attributed to the masking of the active site upon binding of the enzyme to the surface of a mineral particle or to conformational changes, which negatively affect kinetic properties of the enzyme.

Nucleic acids are substrates for soil microorganisms. After adding to soil, DNA or RNA induces an increase in the bacterial viable count. Nucleic acids-soil minerals complexes are intensively colonized by bacteria. The growth of bacteria and fungi is an effect of the nutrients release from nucleic acids by extracellular microbial hydrolases performance.

Moreover, the persistence of the DNA depends on different associations of nucleic acids with soil minerals (Tab. 3).

Lorenz and Wackernagel (1994) noted that transformation-active DNA molecules, although subjected to continual degradation, may persist in soil for weeks or even months. Thus, the persistence of DNA in soil and sediment may rely on the rapid and extensive adsorption of DNA to mineral surfaces, which provides protection against nucleolytic degradation.

Table 3. Half-lives of DNA in various environments (modified from Lorenz and Wackernagel 1994)

Minerals	wt/wt % in sand	$MgCl_2$ conc. (mM) during adsorption	Transformation after incubation with DNase I (%)
Feldspar	12	20	36
Quartz	86.1	20	67
Heavy minerals	1.9	20	32

Free DNA or DNA taken up by cells but not inheritably integrated (because of lack of homology or DNA restriction) is degraded by extracellular and intracellular DNases, respectively (Lorenz and Wackernagel 1994). Soil bacteria actively secrete nucleases into the soil to increase the rate at which the nutrients in soil DNA become accessible (Levy-Booth *et al*. 2007).

Blum *et al.* (1997) reported that DNA degradation begins with the enzymatic restriction of high molecular weight, double-stranded DNA. Briefly, DNA is cleaved into smaller duplex fragments by microbial restriction endonuclease I to about 400 bp oligonucleotides. Oligonucleotides may be degraded to mononucleotides by DNases, which catalyze the hydrolytic cleavage of phosphodiester linkages in the DNA backbone (Mishra 2002). However, such a complete cleavage is not a common process in a natural conditions.

DNA degradation process is strongly affected by temperature. Literature data demonstrated that half-life of extracellular DNA target sequences decreased with an increase of temperature (Gulden *et al.* 2005). Alternatively, DNA degradation slows considerably in frozen soils (Levy-Booth *et al.* 2007).

3.4. Soil pH effect on DNA content

DNA binding to mineral surfaces proceeds over a wide range of pH values, and what is more, the dominant mechanism differs depending on soil pH.

In general, lower pH promotes DNA binding to internal surfaces of soil minerals. DNA content at different soil types under different pH conditions and granulometric composition are presented in Table 4.

Table 4. DNA content in diverse soil types

Soil type	pH	Granulometric content (%)			DNA yield [mg g^{-1} (dry/wt)] of soil	Reference
		Sand	Silt	Clay		
Sandy loam	4.8	54	41	5	8.4 ± 2.8	Zhou *et al*. 1996
Sandy clay	5.8	62	22	16	52 ± 62	Frostegard *et al.* 1999
Sandy clay loam	5.8	34	47	19	60 ± 6.0	Frostegard *et al*. 1999
Clay	4.8	27	26	47	16 ± 8.0	Frostegard *et al*. 1999
Loam	6.4	42	41	17	2.3 ± 0.5	Zhou *et al*. 1996
Loam	8.3	53	_	21	1.32 ± 0.10	Zaporozhenko *et al.* 2006
Silt loam	3.4	30	55	15	138.2 ± 9.8	Bŭrgmann *et al.* 2001

Under the low pH conditions, the phosphate moieties of DNA do not exposed negative charges and, as a consequence displayed decreased repulsion toward net-negatively charged minerals (Crecchio *et al.* 2005).

Also, protonation of the amino groups of adenine, guanine and cytosine occurs, and these positively charged groups are attracted with higher affinity by the pH-independent negatively charged groups of the clay surfaces (Saeki *et al.* 2010). Cai *et al*. (2006) determined that maximum DNA adsorption on montmorillonite, kaolinite, and goethite occurred at around pH 3.0.

At pH 5.0 (approximate isoelectric point for DNA), nucleic acids becomes mineralized and occurs only on the external surfaces of soil minerals, where exchangeable cations are located (Khanna and Stotzky 1992, Franchi *et al.* 1999).

The increase of pH reduces efficiency of cations to mediate adsorption, due to increased electrostatic repulsion between DNA and the soil matrix (Lorenz and Wackernagel 1994).

4. DNA ISOLATION METHODS FROM THE SOIL

Molecular analysis of bacterial diversity in soil environment relies mainly on 16S rDNA amplification, and further DNA analytical techniques (Von Wintzingerode *et al.* 1997). These methods require DNA extracts free from the numerous inhibitory contaminants commonly found in environmental samples (Wilson 1997). What is more, they must exhibit an unbiased sampling of the investigated bacterial community (Robe *et al*. 2003). Selection of appropriate methods for obtaining reliable results could be a promising approach in further genetic investigations.

There are two main approaches of obtaining DNA from the environmental samples. One is the direct extraction of nucleic acids from soil samples after *in situ* cell lysis, which is followed by DNA purification (Ogram *et al.* 1987, Robe *et al*. 2003). The second approach separates the cell fraction from soil particles first. Then, the bacterial fraction is lysed and the nucleic acids are purified (Torsvik 1980, Courtois *et al.* 2001). Both approaches have advantages and disadvantages related to DNA yields.

4.1. Extraction procedures

Extraction methods have been applied to samples taken from various locations. So-called environmental DNA is often the objective in the description and

analysis of the microbial community in the ecosystem. General scheme of microbial DNA extraction methods from environmental samples is shown in Figure 4.

All published extraction techniques derive from the original procedure of Ogram *et al.* (1987). The disruption of microbial cell wall is the first step leading to release of possibly all nucleic acids from bacteria and soil aggregates.

The treatments in this step depend on the (Robe *et al*. 2003):

- cell wall sensitivity to lysis,
- the location of bacteria in microstructures, and
- their interactions with soil particles.

In the second step, nucleic acids are separated from soil particles. DNA isolation includes organic extraction and alcohol precipitation.

Many protocols facilitate breakage by the subsequent deproteination of a soil sample with the addition of CTAB and the increase of salt concentration (Zhou *et al.* 1996, Ranjard *et al*. 1998).

Nucleic acids can be isolated from soil material by the addition of alcohol. The soil extraction protocols used either isopropanol (one volume) or ethanol (two volumes).

Organic extraction with a variety of phenol and chloroform combinations is also included in the extraction protocols to remove a great deal of the humic and pigmental components. In some cases, phenol extraction(s) is followed by chloroform extraction, while at the other a mixture of phenol and chloroform is proposed.

Some protocols (Schneegurt *et al*. 2003) include extracting homogenate supernatants with Tris-buffered phenol and then with water-saturated chloroform : isoamyl alcohol solution (24:1).

In most cases, the mixture is cooled, often overnight before centrifugation to collect nucleic acids. Then, nucleic acids are dried and resuspended in a small amount of buffer (Schneegurt *et al.* 2003). Resuspension can be slow and usually requires heating (65^{o}C for 1 h) or prolonged incubation (overnight at 4^{o}C).

Polisacharides can be removed by PEG precipitation (Porteous and Armstrong 1991). Sometimes, DNA extracts are treated with RNase to remove contaminating RNA as, similarly, RNA extracts are treated with DNase (Nannipieri *et al*. 2003).

Schneegurt *et al.* (2003) indicated that extraction from soils with low organic content is less troublesome. In some cases, extensive dilution of the extract allows the direct PCR amplification from crude DNA extracts.

However, in most protocols the first thing in each extraction procedure – cell lysis (membrane disruption) can be achieved by: physical, chemical and enzymatic disruption or their combination.

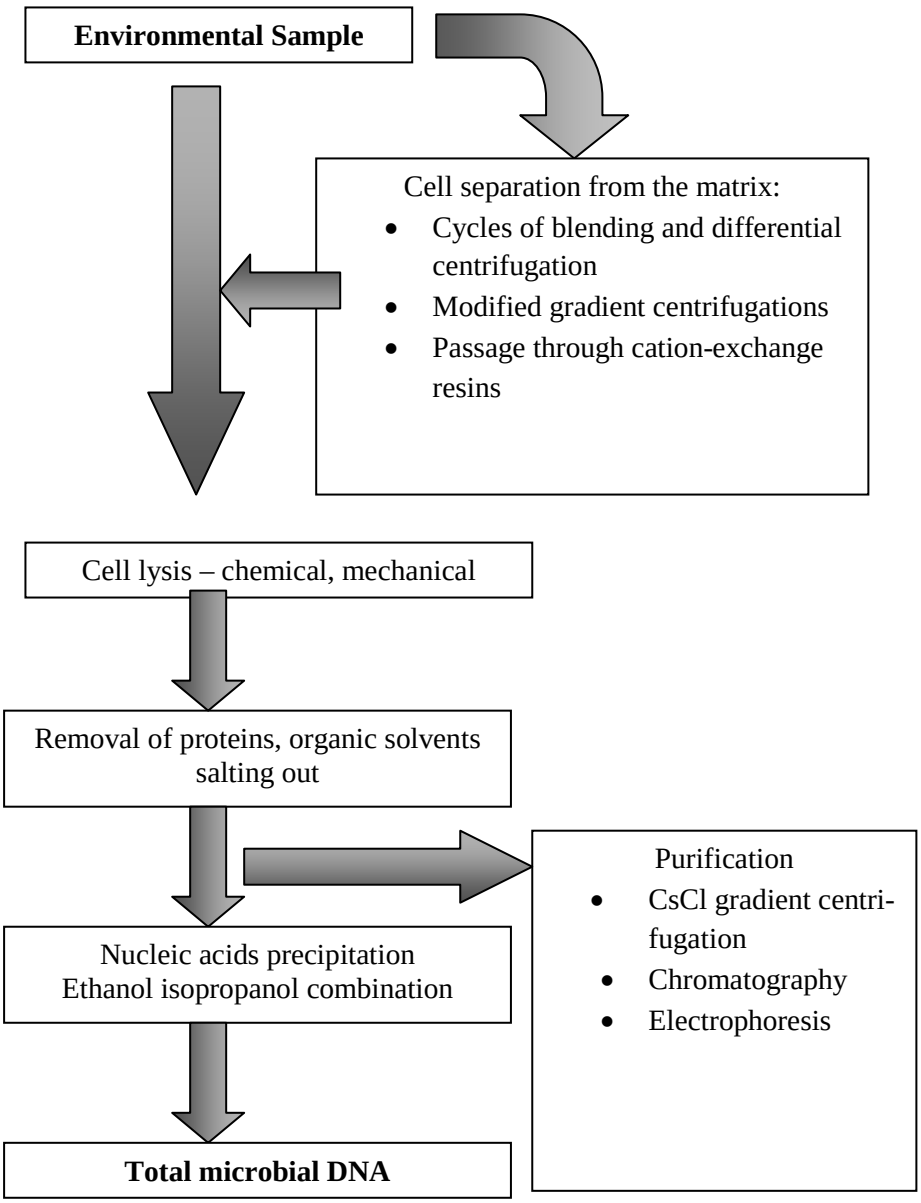

Fig. 4. General scheme of microbial DNA extraction methods from environmental samples (modified from Roose-Amsaleg *et al.* 2001)

4.2. Physical treatments

Physical treatments, which destroy soil structure tend to have the greatest access to the whole bacterial community, including bacteria deep within soil microaggregates.

The most commonly used protocols are based on the one of Tsai and Olsen (1991), where freeze-thaw cycles are used to break the cells before DNA extraction. Soil suspensions are alternately rapidly frozen in a dry ice-acetone, dry ice-ethanol, or liquid nitrogen bath and then rapidly thawed in a 60 to 100^{o}C water bath.

Bead beating is another popular cell breakage method. Soil suspensions are supplemented with the addition of small zirconium/glass beads and then violently shaken at more than 500 rpm until physical cells disruption is achieved (Ogram *et al.* 1987, Tebbe and Vahjen 1993).

Briefly, an equal volume of beads and soil are added to a lysed medium which may contain detergents and high salt concentration. Then, the mixture is milled for 3 to 15 minutes, often in low temperature. However, the size of the beads used the period of milling and the lysis buffer composition vary between protocols (Robe *et al.* 2003).

Methods with use mortar mill grinding (Ranjard *et al.* 1998), ultrasonication (Picard *et al.* 1992) or microwave thermal shock (Orsini and Romano-Spica 2001) have also been reported as commonly used physical cell breakage methods.

The advantage of physical methods is their efficiency in disruption of vegetative forms, small cells and spores, however they often result in significant DNA damage. Therefore, the harsh physical methods can be used in the cases in which fairly small DNA fragments are targeted for amplification and shearing is not as important (Schneegurt *et al.* 2003).

Physical methods are often supplemented with the addition of chemical reagents to enhance the DNA recovery.

4.3. Chemical lysis

Chemical lysis either alone or in association with physical methods has been used extensively.

The most popular detergent treatment includes 1% SDS, which dissolves the hydrophobic material of cell membranes (Porteous *et al.* 1997), but form the other hand can inhibit PCR if not removed in subsequent steps (Schneegurt *et al*. 2003).

Detergent treatments have often been used in combination with heat-treatment and chelating agents, such as EDTA (Jacobsen and Rasmussen 1992), diverse Tris

or sodium phosphate buffers (Schneegurt *et al*. 2003), or PVPP (Picard *et al.* 1992, Frostegard *et al.* 1999).

The function of these chemical agents is to mask positive charge of clay minerals and to chelate multivalent cations in order to increase net repulsion, enhancing detachment of cells and molecules from soil particles (Hopkins and O'Donnell 1992), and hindering reattachment of molecules already released.

Generally, literature data demonstrated that the choice of buffer should be a compromise between the expected amount and purity and DNA quantity.

4.4. Enzymatic lysis

Several protocols using incubation with enzymes have been developed. Lysozyme treatment is one of the most common procedure (Tsai and Olson 1991, Tebbe and Vahjen 1993, Maarit-Niemi *et al.* 2001). Incubation with lysozyme is often proceeded with the addition of other enzymes, i.e. achromopeptidase, protease or proteinase K (Tebbe and Vahjen 1993, Ranjard *et al.* 1998, Courtois *et al*. 2001).

Achromopeptidase is used for the lysis improvement of the recalcitrant Gram-positive bacteria (Simonet *et al.* 1984), whereas proteinase K is used to digest contaminating proteins (Zhou *et al*. 1996, Maarit-Niemi *et al*. 2001, Robe *et al.* 2003, Schneegurt *et al*. 2003).

Krsek and Wellington (1999) indicated that enzymatic lysis method is often insufficient in obtaining satisfying amounts of DNA. Therefore, enzymatic treatment often precedes the addition of detergents and salts in order to help the enzymes to gain access to less efficiently lysed bacteria (Nannipieri and Smalla 2006). Incubation with enzymes combined with other treatments may increase the lysis efficiency, however it should be reported (Robe *et al*. 2003) that a long (an hour or more) and warm (37°C) enzymes digestion may also cause DNA degradation.

Various schemes are, however, invented and tested to gain a sufficient amount of DNA of satisfying purity. Picard *et al.* (1992) used a combination of SDS, enzyme treatment, ultrasonication and microwave treatment to achieve maximal lysis, but the obtained DNA was severely sheared.

4.5. DNA purification

Humic acids have physical and chemical properties similar to those of nucleic acids (Harry *et al.* 1999), so that they can compete with nucleic acids for adsorption site during the extraction step, which may result insufficient purity for further

molecular procedures. That is why, it is necessary to purify samples even if it entails significant DNA losses.

Humic acids inhibits enzyme restriction of DNA during PCR (Tebbe and Vahjen 1993), and also alters the results of quantitative membrane hybridizations by lowering the expected hybridization signal. The phenolic groups in humic acids denature biological molecules by binding to amides or are oxidized to quinine form which covalently binds to DNA (Alm *et al.* 2000).

Purification methods use cesium chloride density gradient ultracentrifugation, chromatography, electrophoresis or more rarely simple dialysis (Romanowski *et al.* 1993, Robe *et al*. 2003).

Other ways of separating DNA from soil impurities include:

- the use of some type of silica gel or silica membrane (Porteous and Armstrong 1991, Zhou *et al*. 1996, Ranjard *et al*. 1998). However, silica gel protocols alone were found to be ineffective in removing sufficient amounts of humic materials from DNA extracts of several soil types (Schneegurt *et al.* 2003),
- the use of agarose gel electrophoresis which allows separation of DNA from humic materials (Herrick *et al.* 1993, Zhou *et al*. 1996),
- membrane-based microconcentrators which can be used for size separations (Porteous *et al*. 1997).

PVPP has also been tested as a purification method (Herrick *et al.* 1993). A strip of gel containing PVPP is often incorporated in a short distance from the wells of an agarose gel. The DNA is unimpeded upon passage through the PVPP strip, while humic materials can be trapped (Schneegurt *et al*. 2003).

Not as popular ion-exchange chromatography was found to be an excellent way to remove humic materials from soil DNA extracts. Torsvik (1980) developed a protocol in which the bacterial lysate was purified by passage through a hydroxyapatite column. DNA was preferentially eluted when the salt concentration of the eluent raised to 0.5 M. Some humic materials were eluted with the DNA, but the vast majority remains bound to the matrix (Schneegurt *et al.* 2003).

There have also been suggested, that differential ethanol precipitations can be used to remove humic materials. Precipitations with 0.1-0.5 volumes of ethanol indeed remove some humic substances (Schneegurt *et al.* 2003). As well, a very traditional method, cesium chloride gradients, appears to be very effective, while purifying soil DNA extracts (Ogram *et al*. 1987, Steffan *et al.* 1988, Courtois *et al.* 2001).

To standardize the extraction procedure, in order to gain the possibility of comparing the obtained results with the results of other scientists, a universal extraction kits were invented. These also give the possibility of obtaining DNA extract without introducing unnecessary bias, which could be caused by taking the sequence of steps. A range of DNA extraction kits which contain purifying agents or provide purification step(s) has been applied to different soil types (Nannipieri and Smalla 2006).

4.6. Commercial kits

Commercial kits are popular and convenient tools that guarantee rapid, safe and efficient DNA isolation from soils samples or rock material.

Commercial kits are designed to isolate ultrapure linear or circular DNA molecules, ranging in size from approximately 100 bp to 10 kb, from TAE – or TBE – agarose gels. It is also possible to purify DNA fragments up to 20 kb or more, with gradually decreasing yields.

Coloured solubilizing buffer helps both in monitoring agarose dissolving and in simultaneous processing of multiple samples. Besides agarose many other contaminants are effectively removed: ethidium bromide, RNA, primers, enzymes and other proteins, lipids, endotoxins, dyes, detergents, nucleotides, radio- and chemical labels, EDTA, problematic restriction and ligation inhibitors, buffers and salts.

Optimized buffer is added to provide selective conditions for DNA binding during brief centrifugation, while contaminants pass through the spin-column. Traces of solubilized agarose and other contaminants remaining on the membrane are efficiently removed in two wash steps. The membrane used is particularly designed toward removal of problematic inhibitors of restriction and ligation of DNA.

High-quality DNA is then eluted in low salt buffer, e.g.: Tris-HCl, TE or water. Isolated DNA is ready for downstream applications without the need for ethanol precipitation.

5. BACTERIA EXTRACTION METHOD

Previously described soil DNA extraction techniques (direct methods) are based on *in situ* lysis of bacteria in soil prior to DNA recovery and purification (Ogram *et al.* 1987, Picard *et al.* 1992).

However, in order to limit mechanical shearing of DNA like contact between DNA and soil components or DNA degradation, a second strategy (indirect method) was developed.

Bacterial extraction was first reported by Faegri *et al.* (1977). Since that time, several other indirect approaches to the extraction of DNA from soil have been published (Holben *et al.* 1988, Jacobsen and Rasmussen 1992).

Bacterial cells are extracted from the soil matrix prior to cell lysis and DNA purification. Anywise, indirect method aims the isolation of only intracellular nucleic acids. This approach typically produces longer DNA fragments.

Indirect extraction of DNA from soil can be advantageous in the assessment of the numbers of specific DNA sequences (targets) present inside bacterial cells. In addition, the extracted bacterial cell pellet can be submitted to other treatments that can increase cloning efficiency and limit contamination by eukaryotic extracellular DNA (Gabor *et al.* 2003, Robe *et al.* 2003).

However, the efficient extraction of microbial fractions from soil is still a major obstacle in most protocols.

Bakken and Lindahl (1995) have used Nycodenz spin tubes and achieved highly efficient separation of bacterial cells from soil.

Jacobsen and Rasmussen (1992) proposed use of Chelex-100 and ion-exchange resin, to dislodge bacterial cells from soil particles.

A rapid protocol, which combined the efficiency of sodium pyrophosphate in resolving bonds in soil aggregates with the efficient cell lysis and DNA extraction and purification provided by the direct method outlined above was also reported by Smalla *et al.* (1993) and Van Elsas *et al.* (1997).

The main disadvantage of cell fractionation-based methods is the fact that the recovered bacterial fraction represents only 25-50% of DNA of the total endogenous bacterial community (Bakken and Lindahl 1995).

In general, bacterial extraction is based on the following sequence of steps (Bakken and Lindahl 1995, Robe *et al.* 2003):

- dispersion of soil particles,
- separation of the cells from soil particles by centrifugation according to sedimentation velocities,

- buoyant density,
- lysis of extracted cells,
- DNA purification.

5.1. Soil dispersion methods

A wide range of physical and chemical dispersion techniques has been used to promote bacterial detachment from soil particles.

Courtois *et al.* (2001) and Lindahl and Bakken (2005) reported that during physical dispersion the use of a warring blender appears to provide reasonable cell yields and simultaneously minimize mechanical cell damage.

Chemical agents which promote dispersion include ligand exchangers and chelating agents, such as oxalate, citrate and EDTA (Katayama *et al*. 1997), Tris buffer (Hopkins and O'Donnell 1992), and pyrophosphate (Lindahl and Bakken 2005).

The intended functions of chemical agents mentioned above (Hopkins and O'Donnell 1992) are:

- masking positive charges of clay minerals,
- chelating multivalent cations in order to increase net repulsion,
- enhancing detachment of cells from soil particles, and
- hindering reattachment of cells already released.

Preservation of bacterial integrity during cell separation seems to be essential in order to prevent released DNA to be degraded by physical, chemical and/or enzymatic processes (Bakken and Lindahl 1995, Ehlers *et al.* 2008).

5.2. Cell separation by centrifugation

Separation of bacteria from soil particles according to sedimentation velocities was first described by Faegri *et al.* (1977). This method is based on two successive centrifugations:

- a low speed centrifugation, ranging from 500 × *g* to 1000 × *g* lasting 2-15 min, respectively, in order to remove soil debris, fungal mycelia and heavy soil particles, and
- a high-speed centrifugation of the cell-containing supernatant produces the bacterial fraction (Robe *et al.* 2003).

An alternative high-speed centrifugation method based on density gradient centrifugation mentioned above was developed by Bakken and Lindahl (1995) in order to separate bacteria according to their buoyant density.

The extraction of bacteria by density gradient centrifugation comprises two steps:

1. to detach bacteria from soil particles by physical or chemical dispersion or by a combination of both,
2. the separation of detached bacteria from soil by high speed centrifugation over a nonionic density gradient medium (Ehlers *et al.* 2008).

Several multi-gradient media have been tested, but the use of Nycodenz provided the best results (Robe *et al.* 2003). Due to differences in buoyant densities, soil particles sink through the Nycodenz medium to the bottom of the centrifugation tube, whereas bacteria float on top of the Nycodenz and can be harvested by siphoning off the supernatant.

Anywise, bacteria attached to soil particles will sink together with soil, hence an imperfect detachment of bacterial cells from soil results in low cell yield and representativeness. The extent of dispersion and detachment of bacterial cells depends on soil type and texture, with lower yields generally observed on soils with higher clay content (Ehlers *et al.* 2008).

6. APPLICATION OF DNA TO MOLECULAR ANALYSES

Most molecular methods routinely used in microbial diversity study are applied on the concept of rRNA phylogeny to the analysis of natural microbial communities. Ribosomal small subunit rRNA genes are now the most commonly used phylogenetic markers to date because of their ubiquity and conserved nature (DeLong and Pace 2001, Leckie 2005).

There are several molecular fingerprinting techniques which provide a rapid assessment of a microbial community. These techniques use PCR amplification, but do not require a clone library. Organisms are separated respectively to the length or sequence polymorphisms, to create a visual pattern, or fingerprint of the community (Leckie 2005).

PCR-based molecular techniques are nowadays powerful methods for surveying the microbial diversity in environmental samples, however, investigators must be aware that such methods can also introduce bias (Roose-Amsaleg *et al.* 2001). They are based on the principle of resolving the diversity of the amplified sequences dependently on their size (ARDRA, t-RFLP) or sequence (DGGE, TGGE) simply by differential electrophoretic migration in agarose or polyacrylamide gels (Tiedje *et al*. 1999, Ranjard *et al.* 2000).

All of these techniques take advantage of 16S rRNA and culture-independent approaches. The initial steps include the extraction of community DNA followed

by the PCR amplification of the 16S rRNA genes from the community DNA using universal, domain or group specific primers.

6.1. Polymerase chain reaction

Beginning in the 1990s, the application of molecular ecological methods, especially those based on surveys of genes after PCR amplification, has allowed cultivation-independent investigations of the microbial communities of soil. The power of these methods has largely rendered obsolete the plate count approach and RNAs has been developed (Gray and Head 2001).

Particularly, 16S rRNA and its gene have proven to be useful and very powerful markers for the presence of bacteria in environmental samples (Rappé and Giovannoni 2003, Janssen 2006).

Anyhow, Janssen (2006) noted that the majority (79-89%) of 16S rRNA gene sequences are from bacteria that are not affiliated with known genera.

Everett *et al*. (1999) suggested that 16S rRNA gene sequence similarities of <96% are indicative of the hosts of the genes belonging to different genera.

PCR technique was introduced in 1983 by Kary Mullis, and since that time is a common and often indispensable technique used in medical and biological research labs for a variety of applications.

The main purpose and usefulness of a PCR is to make a large number of copies of a gene. This is necessary to have enough starting template for sequencing or different molecular analyses. There are three major stages in a PCR, which are repeated for 30 or 40 cycles (Fig. 5).

1. **Denaturation** – the aim of this step is to made that the double strand melts open to single stranded DNA.
2. **Annealing** – the step when the primers(both forward as reverse) are jiggling around, caused by the Brownian motion. Moreover, ionic bonds are now formed and the DNA-polymerase could attach and begin copying the template. The temperature used in this stage is not constant and depends from our starters sequences.
3. **Extension** – the last step of PCR, when the bases are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template).

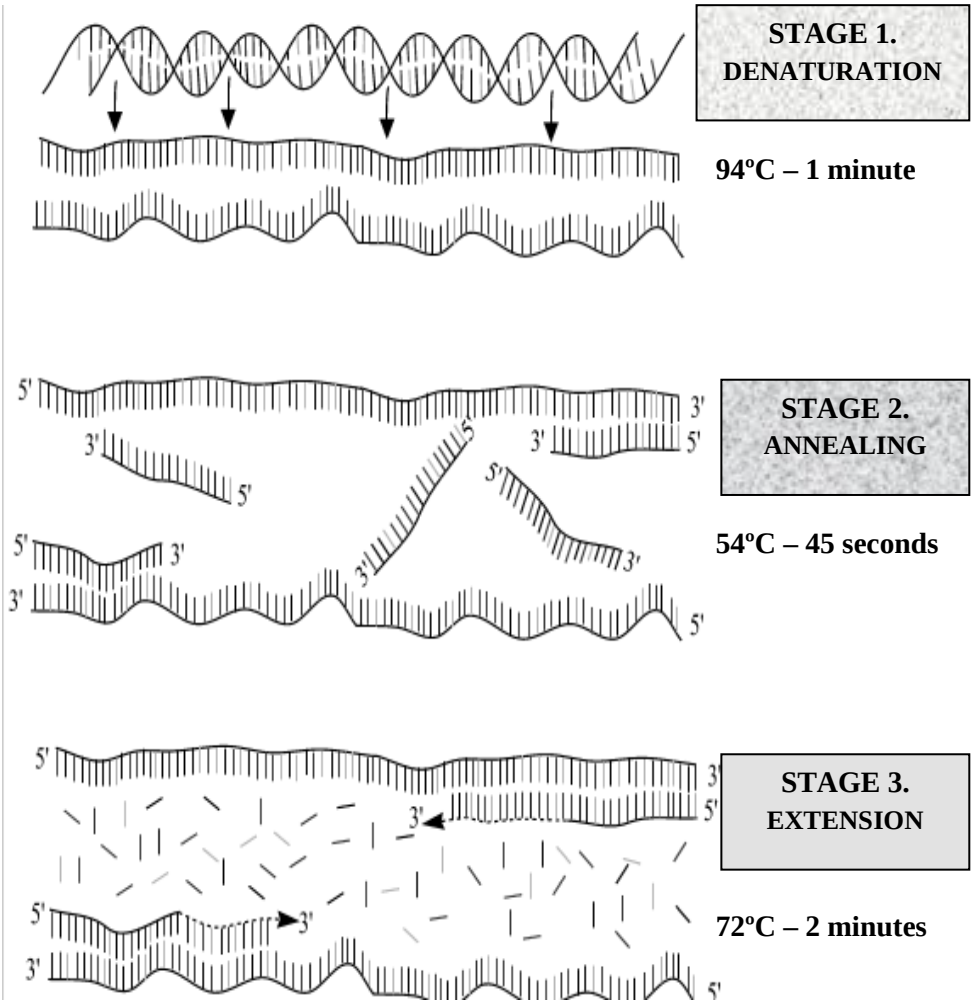

Fig. 5. The difference steps in PCR (according to Vierstraete 1999)

It should be remember that, before the PCR product might be used in further applications, it has to be necessary checked if (Fig. 6):

- there is a product formed (as not every PCR is successful). There is a possibility that the quality of the DNA is poor, or that one of the primers does not fit, or that there is too much starting template,
- the product is of the expected size,
- only one band is formed.

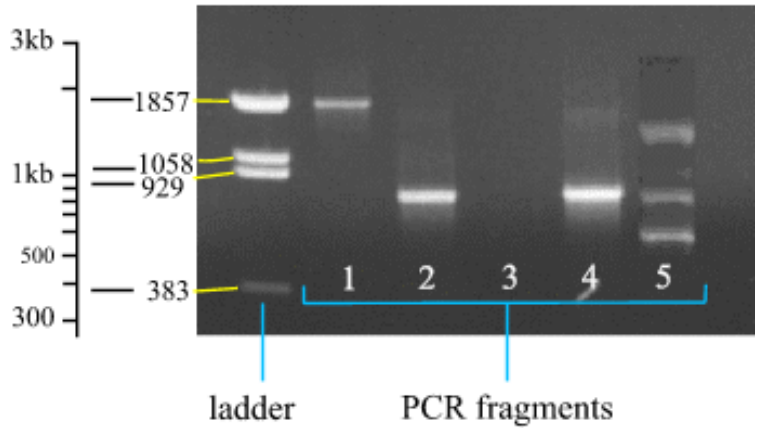

Fig. 6. Verification of the PCR product on gel (sample taken from Vierstraete 1999). The ladder is a mixture of fragments with known size to compare with the PCR fragments. Notice that the distance between the different fragments of the ladder is logarithmic. **Lane 1** : PCR fragment is approximately 1850 bases long. **Lane 2** and **4** : the fragments are approximately 800 bases long. **Lane 3** : no product is formed, so the PCR failed. **Lane 5** : multiple bands are formed because one of the primers fits on different places

The original method of PCR used the Klenow fragment of *E. coli* DNA polymerase I (Saiki *et al.* 1985). This enzyme, however, denatures at temperatures lower than that required to denature most template duplexes. Thus, in nowadays the commonly use of heat-resistant DNA polymerase obviously facilitated the process, because the addition of enzymes after every denaturation cycle is no longer necessary.

The first thermostable DNA polymerase used was the *Taq* DNA polymerase isolated from the bacterium *Thermus aquaticus* (Saiki *et al.* 1985). Currently, several other DNA polymerases are also commercially available (Newton and Graham 1994), among them the most popular are:

- Vent™ -; DeepVent™-; Pfu- and UITma™- DNA polymerases - these enzymes have a 3'- 5' exonuclease activity, which cause the removal of mismatched residues until a correctly base-paired terminus is generated. However, we should realize that mentioned exonuclease activity could effect on degradation of the primers. Therefore, the enzyme should only be added after the reaction has started.
- AmpliTaqGold™- DNA polymerase - enzyme consists of an Ampli*Taq* DNA polymerase, inactive at room temperature, and can only be activated during an incubation period at 94^{o}C.

Perhaps the most critical parameter for successful PCR is the proper designing of primers. All things being equal, a poorly designed primer can result in a PCR reaction that will not work.

The primer sequence determines several things like the position and length of the product, its melting temperature and ultimately the yield (Innis and Gelfand 1994). Sambrook *et al.* (1989) indicated that primers need to be designed with absolutely no intra-primer homology beyond 3 base pairs. If a primer has such a region of self-homology, "snap back", or "hair-pin", partially double-stranded structures can occur, which will interfere with annealing to the template.

Generally, PCR mixture for amplification reaction should consists of the following components (Agnelli *et al.* 2004): 1 µl of each primer (10 µM), 1 U of Taq DNA polymerase, 5 µl $MgCl_2$ (0.5 mM), 1 µl BSA (500 µg ml^{-1}), 1 µl 10mM dNTP's and 3 µl of DNA extracted. However, the presence of BSA in PCR mixture is obligatory, it is recommended to use it as BSA has had widespread use for relieving interference in PCR (Kreader 1996).

All reagents (except the template DNA) are mixed in a single tube, in enough volume according to the number of reactions to be performed (mastermix).

The presence of divalent cations in PCR mixture is critical. The $MgCl_2$ concentration is recommended to be usually between 0.5 to 5.0 mM, however the optimum concentration is determined empirically (Innis and Gelfand 1994). Mg divalent cations have ability, both, for to forming a soluble complex with dNTPs which is essential for dNTP incorporation, as for stimulation of polymerase activity.

Free dNTPs are required for DNA synthesis. It concentrations for PCR should be 20 to 200 µM for each dNTP and the four dNTPs should be used at equivalent concentrations to minimize misincorporation errors (Innis *et al*. 1988). It is also recommended that dNTPs stock solutions (usually 100 mM) should be adjusted to pH 7.0-7.5 with 1 M NaOH to ensure that the pH of the final reaction does not fall below 7.1 (Sambrook *et al.* 1989) however, many dNTPs stock solutions are now supplied with already adjusted pH.

6.1.1. Nested – PCR

Newton and Graham (1994) described that nested sets of primers can be used to improve PCR yield of the target DNA sequence. PCR with nested primers is performed for 15 to 30 cycles with one primer set and then for an additional 15 to 30 cycles, with a second primer set, for an internal region of the first amplified

DNA product. As an effect, the larger fragment produced by the first round of PCR is used as the template for the second PCR cycle.

During the second round of amplification the annealing temperature is lowered so that the inner pair of primers can function. Due to the fact that in this method multiple primer pairs are used, the increase of possibility primer-primer interaction should be very carefully considered.

The easiest way to avoid mentioned interaction is to use the reduced length of the nested (inner) primers, as compared to outers primers (18-20 bases versus 25-28 bases). What is more, the inner primers should be used in excess (about 40 times more) than outers primers.

The specificity and sensitivity of nested-PCR method is particularly enhanced, because this technique almost always eliminates any spurious non-specific amplification products. However, the increased risk of contamination is a drawback of this extreme sensitivity, and great care must be taken when performing such PCRs type.

On the other hand nested-PCR technique can detect target DNA at several-fold lower concentrations than conventional PCR (Fan *et al.* 2009). Thus its use is recommended when the DNA content in samples is low.

Nested PCR has been used to investigate the microbial diversity of samples such as arctic sea-ice (Gerdes *et al.* 2005), marine sponges (Li *et al.* 2006), Paleolithic paintings and surrounding rock walls (Schabereiter-Gurtner *et al.* 2004).

6.1.2. Multiplex - PCR

Multiplex PCR uses multiple pairs of primers to amplify many sequences simultaneously. Typically, primer concentrations, salt concentrations, and annealing temperatures are adjusted in an effort to balance the annealing rates of all the primers in the reaction. Unfortunately, as the number of amplicons in a PCR is increased, it becomes more difficult, if impossible, to work out conditions to obtain an equal amount of each product.

The presence of many PCR primers in a single tube could cause many problems, such as the increased formation of mispriced PCR products, "primer dimmers", and the amplification discrimination of longer DNA fragments (Atlas and Bey 1994). For this type of PCR amplification, primers must be chosen according to similar annealing temperatures. Thus, a multiplex PCR that yields equivalent amounts of each PCR product can be difficult and laborious.

Anyway, the expense of reagents and preparation time is shorter in multiplex PCR than in system where several tubes of uniplex PCRs are used. For this reason a multiplex reaction is ideal for conserving expensive polymerase and templates in short supply (Edwards and Gibbs 1994). The main six steps of multiplex PCR are outlined below:

1. Choose Loci
    - determine PCR system,
    - distribute amplicons (localized at mutation hot spots, linked to genes, chromosomally unliked etc.),
    - design internal control fragments (other exons, external sequences, host sequence etc.).
2. Position primers in regions of detailed sequence, in relation to amplicon sizes.
3. Design primers with similar reaction kinetics.
4. Develop PCR conditions separately for each primer set.
5. Add primers set sequentially
    - alter conditions as necessary,
    - reduce nonspecific amplifications (hot starts, ionic detergents, short extension times, hottest annealing, reselect primer sequence),
    - change buffer system if necessary.
6. Adjust reaction components and cycling conditions for multiplex amplification.
    - remember that Mg^{2+}, dNTPs, and polymerase requirements may increase,
    - ideal extension times may be longer.

Multiplex PCR products can be further hybridized with a gene-specific probe for verification. If the multiplex product is to be resolved electrophoretically, fragments sizes should be selected so that they might be separated easily form each other (Edwards and Gibbs 1994). However, with the use of fluorescently labeled primers, product ranges may overlap and yet be distinguished by color. Edward and Gibbs (1994) demonstrated that fluorescently-labeled multiplex primers are helpful in diagnostic studies by representing product amounts more accurately than ethidium bromide stain and by reducing reaction time and nonspecific amplification with the fewer PCR cycles needed to obtain a signal.

6.1.3. Real time – PCR

In recent years real-time PCR (also referred as qPCR) has emerged as a promising tool for studying soil microbial communities (Fierer *et al*. 2005). These technique is based on the real-time detection of a reporter molecule whose fluo-

rescence increases as PCR product accumulates during each amplification cycle (Raeymaekers 2000).

Fierer *et al*. (2005) reported that real-time PCR approach is somewhat unique among methods of community analysis in that it allows for a relatively rapid yet quantitative assessment of the abundances of specific phylogenetic groups of microorganisms in soil.

A group-specific primers the most popular in real time-PCR, as they are referred on the main groups of microorganisms inhabiting the soil environment are presented in Table 5.

Table 5. Primers used for real-time PCR assays, annealing temperatures, target regions, and the specificity of the amplicons cloned from real-time PCR assays with soil DNA (according to Fierer *et al*. 2005)

Target group	Forward primer	Reverse primer	Approxi-mate amplicon length (bp)	Annea-ling temp. (ºC)	% of soil clones belonging to the target groups
All Bacteria	Eub338	Eub518	200	53	100
α Proteo-bacteria	Eub338	Alf685	365	60	75
ß-Proteo-bacteria	Eub338	Bet680	360	60	96
Actino-bacteria	Actino235	Eub518	300	60	60
Firmicutes	Lgc353	Eub518	180	60	100
Bacteroidetes	Cfb319	Eub518	220	65	100
Acidobacteria	Acid31	Eub518	500	50	100
All Fungi	5.8s	ITS1f	300	53	100
Basidiomycota	ITS4b	5.8sr	500	55	100

Most of published literature data demonstrated that *Acidobacteria*, *Proteo-bacteria*, *Actinobacteria* and *Bacteroidetes* are generally the numerically dominant phyla in soils, with members of *Firmicutes* being less common (Dunbar *et al*. 2002, Winding *et al*. 2005, Fierer *et al.* 2006).

A significant limitation of real-time PCR is that the estimated abundances of the different microbial groups may not equal the true percentages of these groups in the soil samples (Fierer *et. al.* 2005).

There are number of reason for mentioned limitations:

- DNA extraction bias may alter the estimated abundances of certain groups Martin-Laurent *et al.* 2001),
- heterogeneity in ribosomal operon number (Tourova 2003), may affect relative estimations of group abundances,
- the tested real-time PCR do not necessarily amplify rRNA genes belonging to all members of each targeted group (Fierer *et al.* 2005).

Summarizing, the real-time PCR approach could be well adapted for providing more comprehensive assessments of soil microbial community structure. To do so, designing the appropriate oligonucleotide primers set and optimalization the PCR conditions reaction are necessary. The flexibility, ease of use, and quantitative nature of the real-time PCR method make it valuable tool for characterizing microbial communities (Fierer *et al.* 2005).

6.1.4. Reverse transcriptase – PCR

RT-PCR is an another variant of traditional PCR technique, however in this method an RNA strand is first reverse transcribed into its complement DNA (cDNA) using the enzyme, reverse transcriptase.

RT-PCR utilizes a pair of primers complementary to a defined sequence on each of the two strands. These primers are then extended by a DNA polymerase and a copy of the strand is made after each cycle, leading to exponential amplification (Hunt 2006).

RT-PCR includes three major steps:

- reverse transcription (RT) – stage when RNA is reverse transcribed to cDNA using reverse transcriptase. This step can be performed either in the same tube with PCR (one-step PCR), or in separate ones (two-step PCR) using a temperature between 40°C and 50°C, depending on the properties of the reverse transcriptase used (Bustin 2000).
- the denaturation of the dsDNA at 95°C – the second step leading to the two strands separation and the moment when primers can bind again at lower temperatures and begin a new chain reaction. Then, the temperature is decreased until it reaches the annealing temperature (Innis *et al.* 1994).
- DNA extension from the primers – final step of RT-PCR reaction, which is done with thermostable Taq DNA polymerase, usually at 72^{o}C, the temperature at which the enzyme works optimally.

The RT-PCR is generally performed with the One-Tube RT-PCR kit (i.e. Roche Molecular Biochemicals, Indianapolis, Ind., or Applied Biosystems, Fermentas etc.) by using 5 µl of RNA and 50 pmol of each primer in a 50-µl total reaction volume with the following cycling times and temperatures: 1 cycle of 45^{o}C for 1 h and 94^{o}C for 3 min and 40 cycles of 94^{o}C for 30 s, 55^{o}C for 1 min, and 68^{o}C for 3 min. After that RT-PCR product in a quantity of a 5-µl should be analyzed by agarose gel electrophoresis (Lanciotti *et al.* 2000), and the DNA should be visualized by ethidium bromide staining.

However, we should be aware that RT-PCR is a time-consuming technique with important limitations when compared to i.e. real-time PCR methods (Mackay and Nitsche 2002).

This fact is combined with the opinion that commonly used ethidium bromide has low sensitivity, so yields results that are not always reliable. More sensitive and recommended are i.e. SYBR Gold or SYBR Green (Invitrogen). What is more, there is an increased cross-contamination risk of the samples since detection of the PCR product requires the post-amplification processing of the samples. Then again, the specificity of the assay is mainly determined by the primers, which can give false-positive results.

Anyhow, the most important issue concerning conventional RT-PCR is the fact that it is a semi- or even a low-quantitative technique, during which the amplicon can be visualized only after the amplification ends.

Moreover, the advantage of mentioned technique is the fact, that exponential amplification via reverse transcription PCR provides for a highly sensitive technique in which a very low copy number of RNA molecules can be detected. However, it should be taken into account that RT-PCR investigations (RNA isolation, cDNA synthesis) demand significantly higher purity in laboratory (i.e. tips filtration, RNase removal from surfaces applied).

Reverse transcriptase-PCR has been commonly used to develop highly sensitive and specific assays for the identification of several RNA viruses (Lanciotti *et al*. 2000). RT-PCR is also widely useful in the diagnosis of genetic diseases and, semi quantitatively, in the determination of the abundance of specific different RNA molecules within a cell or tissue (Hunt 2006). Thus, RT-PCR is considered as one of the most widely applied techniques in biomedical research, and has been a major boon to the molecular investigation of disease pathogenesis.

6.2. Electrophoresis

The powerful tool of DNA analysis gel electrophoresis, was developed in the 1970s, as an analytical technique used to separate an isolated DNA fragments by size. DNA molecules are set into a medium - the gel (agarose, polyacrylamide), where an electric field induces the DNA to migrate towards the anode, due to the net negative charge of the sugar-phosphate backbone of the DNA chain. The separation of DNA fragments is strictly accomplished by exploiting its ability for mobile.

Longer molecules migrate slower because they experience higher resistance within the gel, whereas smaller fragments end up nearer to the anode than longer ones in a given period. After some time, the voltage is removed and the fragmentation gradient is analyzed. For larger separations between similar sized fragments, either the voltage or run time could be increased.

The DNA fragments of different length are visualized using a fluorescent dye specific for DNA, the most common is ethidium bromide. DNA fragment size is usually reported in "nucleotides" or "base pairs", depending upon whether single- or double-stranded DNA has been separated. Capillary electrophoresis results are typically displayed in a trace view called an electropherogram.

TAE buffer is historically the most common buffer used for agarose gel electrophoresis, due to its low ionic strength and low buffering capacity. It is the best suited to electrophoresis of large (>20 kb) pieces of DNA and will need to be replaced frequently or recalculated for longer (>4 h) gel run times.

Another popular buffer is TBE, which has a greater buffering capacity and will give sharper resolution than mentioned above TAE buffer. However, TBE is generally more expensive than TAE, and inhibits DNA ligase which may pose problems if subsequent DNA purification and ligation steps are intended.

The exemplary electropherogram is shown in Fig. 7. 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 (Wolińska 2009). PCR products were visualized with ethidium bromide (0.25 µg l^{-1}).

Electrophoresis is considered to be the main technique for molecular separation in today's cell biology laboratory. Because it is such a powerful technique, and yet reasonably easy and inexpensive, it has used commonly in many laboratories.

Nowadays with developing molecular tools, also traditional electrophoresis gained some modifications like presented below techniques: DGGE/TGGE or SCCP.

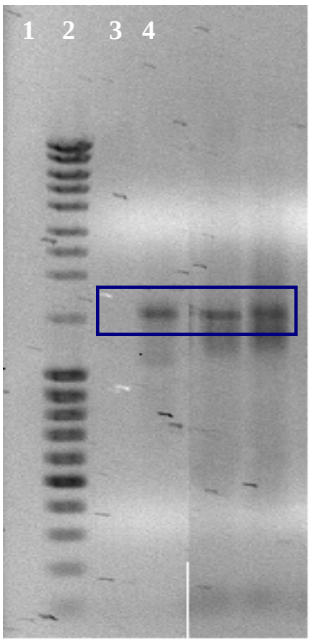

Fig. 7. Electropherogram of PCR products, **1:** MassRuler™ DNA Ladder, **2:** DNA fragment isolated from *Orthic Podzol* sample, **3:** DNA from *Rendzina Leptosol*, **4:** DNA from *Haplic Phaeozem* (according to Wolińska 2009)

6.2.1. Denaturing gradient gel electrophoresis

DGGE technique was first applied to bacterial communities by Muyzer *et al*. (1993) and is now commonly used.

The method involves extracting total community DNA from soil, amplifying a short region of the 16S rRNA gene that differs in sequence among organisms in the community, and resolving the mixture of gene fragments based on differential DNA dissociation (or ‘‘melting’’) behavior.

Fragments migrate through a gel matrix with an electric current along a gradient of increased concentration of DNA denaturant. Once a fragment reaches a concentration of denaturant sufficient for denaturing to begin, the double strands start to separate and this causes the fragment to cease migrating.

The fingerprint is then comprised of different fragments migrating to different points in the gel. Amplified samples with different sequences are separated during electrophoretic migration, yielding the complex profiles representing the diversity of the fragments amplified (Ranjard *et al*. 2000). Muyzer *et al.* (1993) expanded the use of DGGE to study microbial genetic diversity. Theoretically, DGGE can separate DNA with one base-pair difference (Miller *et al.* 1999, Kirk *et al.* 2004).

6.2.2.Thermal gradient gel electrophoresis

Both DGGE as TGGE were originally developed to detect point mutations in DNA sequences. TGGE has been successfully used to assess the diversity of bacteria and fungi in the rhizosphere (Smalla *et al*. 2001), and its changes caused by addition of anthropogenic chemicals (Torsvik *et al*. 1998, Whiteley and Bailey 2000).

Theron and Cloete (2000) revealed, that additional advantage of TGGE bands is that they can be excised from gels, re-amplified and sequenced or transferred to membranes and hybridized with specific primers to provide more structural or functional diversity information.

However, limitations of DGGE/TGGE methods include:

- PCR biases (Wintzingerode *et al*. 1997),
- variable DNA extraction efficiency (Theron and Cloete 2000),
- laborious sample handling (including calibration of the linear gradient of DNA denaturants and improvement of the PCR primers with the insertion of a G+C clamp) to obtain better electrophoretic separation of the fragments, as this could potentially influence the microbial community (Ranjard *et al*. 1998, Theron and Cloete 2000, Kirk *et al.* 2004),
- possibility of some genotype founding in one stripe, if melting temperature is the same for different sequences.

6.2.3. Single strand conformation polymorphism

Another technique that relies on electrophoretic separation based on differences in DNA sequences is single strand conformation polymorphism. Similarly to DGGE/TGGE, presented method was originally developed to detect known or novel polymorphisms or point mutations in DNA sequences (Orita *et al.* 1989).

Single-stranded DNA is separated on a polyacrylamide gel based on differences in mobility caused by their folded secondary structure (Lee *et al.* 1996).

When DNA fragments are of equal size and no denaturant is present, folding and hence mobility, is dependent on the DNA sequences.

The SSCP technique may be useful to develop probes that detect changes in the microbial community caused by an environmental change. It has also been used to study bacterial or fungal community diversity (Stach *et al.* 2001), or has been used for identification of bacteria since it provides a genomic fingerprint of chromosome structure.

The chromosome structure is considered to be variable between strains (Tiedje *et al*. 1999). Many organisms, both prokaryotic and eukaryotic, contain highly repetitive short DNA sequences that are 1–10 bp long repeated throughout their genomes (Longato and Bonfante 1997, Tiedje *et al.* 1999).

SSCP has all the same limitations as DGGE. Furthermore, it should be considered that some single-stranded DNA can form more than one stable conformation. Therefore, one sequence may be represented by more than one band on the gel (Tiedje *et al.* 1999, Kirk *et al.* 2004).

However, this technique does not require a G+C clamp or the construction of gradient gels. Nevertheless, the use of this method to study microbial diversity may also be limited dependently on the complexity of the community.

6.3. Molecular-based techniques to study microbial diversity

During the last few years, to study the distribution and activity of microorganisms in the environment, microbial ecologists have switched more and more to molecular strategies (Liu *et al.* 2006).

A number of approaches have been developed to detect microbes in soils, such as G+C content, nucleic acid reassociation and hybrydization, RFLP/T-RFLP, RISA/ARISA spacer analysis, RSGP, CLPP, PLFA and FISH. Each of recollected above techniques are shortly summarized below this chapter.

6.3.1. G+C content

Presented method was described in detail by Holben and Harris (1995). This technique is based on the fact that prokaryotic DNA varies in G+C content from 24% to 76% G+C *vs.* A+T and that particular taxonomic groups only include organisms which vary in G+C content by no more than 3±5% (Vandamme *et al.* 1996). The % G+C distribution is sensitive to changes in the composition of the microbial community because different species carry different proportions of % G+C. (Holben and Harris 1995) so simple interpretation of % G+C is equivocal.

Generally, the separation by base composition is based on the principle that bisbenzimidazole is preferentially bound to A+T base pairs, thus amplifying the differences in the gravity of the DNA according to the specific G+C content (Kirk *et al.* 2004). The extracted DNA is centrifuged through a cesium chloride density gradient in the presence of bisbenzimidazole. This yields a DNA community profile of % G+C (base composition) values providing a molecular fingerprint of the overall community structure (Ranjard *et al.* 2000).

Holben and Harris (1995) demonstrated, that the majority of DNA extracted from cultivated soil corresponds to the % G+C of range 55–73, which includes bacterial genera known to be abundant in soil communities under field conditions. The main advantages of the G+C method are as follows (Tiedje *et al.* 1999):

- it is comprehensive for all DNA extracted;
- it is not subject to the biases of PCR-based methods,
- the portions of the DNA are not missed in analysis due to ineffective hybridization or similar losses in the analysis,
- it is quantitative and it can uncover rare members in the microbial populations. It does, however, require large quantities of DNA.

However, we should realize that the method is a coarse measure of resolution since several taxonomic groups, containing different species, could co-incidentally have similar % G+C profiles and in the case distinct peak, different organisms could also contribute to a single peak, thus, analysis of G+C content is useful when a level of resolution is meaningful (Holben and Harris 1995, Tiedje *et al.* 1999).

In microbiology, most methodologies provide a medium-to-fine-scale resolution, i.e. genus to species to subspecies level (Vandamme *et al.* 1996). Hence, the % G+C method fills a gap in the tool box by providing one of the few coarse-level methods, especially for community analysis.

6.3.2. Nucleic acid reassociation and hybridization

DNA reassociation according to Torsvik *et al.* (1990) is a measure of genetic complexity of the microbial community and has been used to estimate microbial diversity.

Total DNA is extracted from environmental samples, purified, denatured and allowed to reanneal (Kirk *et al.* 2004). This technique involves the heat denaturation of DNA followed by reassociation of the homologous single strands. The proportion of DNA renatured is generally expressed as the function of the product

concentration of nucleotides and the reaction time (Theron and Cloete 2000). Thus, the rate of reassociation depends most of all on the similarity of sequences present. As the complexity or diversity of DNA sequences increases, the rate at which DNA reassociates decreases (Theron and Cloete 2000).

Under specific conditions, the time needed for half of the DNA to reassociate (the half association value) can be used as a diversity index (Torsvik *et al.* 1998).

DNA hybridization is a technique where two samples of DNA community are hybridized against each other in a reciprocal manner (i.e. sample A as probe against sample B as target, and sample B as probe against sample A as target).

Hybridization technique has been used to determine the similarity (percentage of DNA in common) between different communities from the environment (Griffiths *et al.* 1997). The extent to which the radiolabelled probe anneals to the filter-bound target DNA reflects the similarity of probe and target and consequently the extent to which the population structure of bacterial communities is similar (Ranjard *et al.* 2000).

The hybridization method can be done on extracted DNA or RNA. Oligonucleotide or polynucleotide probes of known sequences ranging in specificity from domain to species can be tagged with markers at the 5-end (Theron and Cloete 2000).Traditionally, radioactive isotopes were used to label oligonucleotide probes, but recently fluorescent probes are often preferred (Kirk *et al.* 2004).

Hybridization can also be conducted *in situ*. This provides valuable spatial distribution information on microorganisms in environmental samples. Therefore, nucleic acid hybridization using specific probes is an important qualitative and quantitative tool in molecular bacterial ecology. For instance, using this method Torsvik *et al*. (1990) estimated, that the number of different bacterial genomes present per gram of soil ranged from 350 to 10 000 according to soil type. Similar findings were presented as well by Griffiths *et al.* (1997).

6.3.3. Restriction fragment length polymorphism

RFLP, also known as amplified ribosomal DNA restriction analysis (ARDRA) is a tool used to study microbial diversity, that relies on polymorphisms of DNA.

In general, this approach has been used most frequently on isolates as part of a clone screening step prior to sequencing or, in some cases, to provide a level of insight into phylogeny. More recently, the technique has been used to probe community structure (Tiedje *et al.* 1999).

RFLP approach does not work well in soils with highly diverse and non-dominant populations, since too many bands are produced to be resolved. This technique has been used rather to demonstrate changes in the genetic structure of bacterial communities, following changes in environmental conditions or exposure to exogenous toxic compounds but not as a measure of diversity or detection of specific phylogenetic groups (Liu *et al*. 1997).

The main advantage of this method is its convenience, because it does not require any particular equipment (Tiedje *et al*. 1999, Ranjard *et al.* 2000). However, in a complex community with many different species, where a single species can contribute four-to-six restriction fragments to the community pattern, it quickly becomes apparent that a RFLP/ARDRA profile is too complex and consequently, loses the information that is important in community analysis (Tiedje *et al.* 1999, Roose-Amsaleg *et al.* 2001).

6.3.4. Terminal restriction fragment length polymorphism

For the purpose of T-RFLP method DNA is obtained by PCR amplification, where universal fluorescently tagged primers are used and detected by an automated sequencer.

We should remember that, only the terminal restriction fragment can be detected and the amount can be quantified using the sequencer. Thus, PCR primer is earlier labeled with a fluorescent dye, such as: TET or 6-FAM. This guarantees the detection of only the labeled terminal restriction fragment (Liu *et al.* 1997). Fragments are resolved by size on polyacrylamide gels using an automated analyzer with laser detection of the terminally labeled products, producing a highly reproducible fingerprint of the community (Fierer and Jackson 2006).

Restriction fragment lengths can be determined for the entire ribosomal database and, therefore provide a logical phylogenetic starting point (Tiedje *et al.* 1999). Thus, allows the analysis of complex communities and also provides information on diversity as each visible band represents a single operational taxonomic unit or ribotype (Tiedje *et al.* 1999). For example, this technique has been successfully used for characterization of bacterial and fungal communities in grassland or forest soils (Dickie *et al.* 2003, Leckie 2005).

T-RFLP method may be limited by DNA extraction, PCR biases, and the choice of universal primers. None of the presently available universal primers can amplify all sequences from *Eukaryota*, *Bacteria* and *Archaea* domains. Moreover, T-RFLP does not underestimates total bacterial diversity because the method re-

solves only a limited number of bands per gel (generally <100), and bacterial species can share phylotypes (Fierer and Jackson 2006).

Additionally, these primers are based on existing 16S rRNA, 18S rRNA or ITS databases, which until recently contain mainly sequences from cultivable microorganisms, and therefore, may not be representative of the true microbial diversity in a sample.

Only numerically dominant species are detected as a result of the large quantity of available template DNA. In addition, different species have different gene copy numbers and this also can bias the results (Liu *et al.* 1997).

However, the method does provide a robust index of bacterial diversity, and T-RFLP results are generally consistent with the results from clone libraries (Fierer and Jackson 2006).

6.3.5. Ribosomal and automated ribosomal intergenic spacer analysis

RISA and ARISA techniques, similar in principle to RFLP and T-RFLP, provide ribosomal-based fingerprinting of the microbial community.

In RISA and ARISA, the IGS region between the 16S and 23S is amplified by PCR, denatured and separated on a polyacrylamide gel under denaturing conditions. This region may encode tRNAs and is useful for differentiating between bacterial strains and closely related species because of heterogeneity of the IGS length and sequences (Fisher and Triplett 1999). In RISA, the sequence polymorphisms are detected using silver stain while in ARISA the forward primer is fluorescently labeled and is automatically detected (Fisher and Triplett 1999).

Both methods provide highly reproducible bacterial community profiles but RISA requires large quantities of DNA and is more time-consuming. Silver staining is somewhat insensitive and resolution tends to be low (Fisher and Triplett 1999).

Several works have demonstrated the reproducibility and the capacity of this approach to detect modifications in community structure between different soil types and even between closely related communities, such as those associated with various microenvironments of soil (Ranjard *et al*. 2000).

RISA and ITS community profiles are similar but involve fragments of DNA from spacer regions separating RNA genes of bacteria and fungi, respectively. Because the spacer regions do not code a product, they are highly variable and can be resolved on a polyacrylamide gel according to their length polymorphism. These techniques, though used less often, have shown to give reliable fingerprints of complex bacterial and fungal communities (Ranjard *et al*. 2001, Leckie 2005).

6.3.6. Reverse sample genome probing

Reverse sample genome probing is a method used to analyze microbial community composition of the most dominant cultivable species, which uses genome microarrays. RSGP has the following steps:

- isolation of genomic DNA from pure cultures,
- cross-hybridization testing to obtain DNA fragments with less than 70% cross-hybridization. DNA fragments with greater than 70% cross-hybridization are considered to be the same species.

This method has been used to analyze microbial communities in the contaminated soils (Voordouw *et al.* 1993, Greene *et al.* 2000).

The use of RSGP has the advantage, as it is not influenced by PCR biases. However, it only detects the most abundant species. In general, the species need to be cultured, but in principle cloned DNA fragments of unculturables could be used (Kirk *et al.* 2004).

Methods, based on the analysis of nucleic acids provide the first step for a more targeted and detailed analysis of composition and diversity. Techniques to describe the complex microbial communities are being developed in contrast to cultural techniques which are both selective and unrepresentative for the total community.

6.3.7. Community level physiological profile

CLPP method is the way of microbial community determination by use of multiwell plates of Biolog Inc. (Garland and Mills 1991).

A soil extract is incubated on these plates with up to 95 different carbon sources and with patented nutrient and salt solution in addition to a redoxdye (Winding *et al.* 2005). In general, color development over time is measured and the degree of loss of specific features after stepwise dilution of the community is regarded as indicative of the CLPP and used as input data for multivariate statistical analysis (Winding *et al.* 2005).

Preston-Mafham *et al*. (2002) demonstrated different types of plates which are available to study bacterial and fungal communities.

The CLPP is strongly limited and dependent on growth of cells under specific conditions in the Biolog plate (Winding *et al.* 2005). Thus, a critical step in the procedure for CLPP is preparation of the microbial inoculum from the environmental samples.

Winding and Hendriksen (1997) indicated that prediction of the exact responses on Biolog plate from cell density, or even from biomass is impossible, as the relation between inoculum density and the response in Biolog plates is not linear.

Hence, highly recommended is use of series of dilutions of the microbial communality for purpose of inoculation of a series plates (Boivin *et al.* 2002, Rutgers and Breure 1999).

6.3.8. Phospholipid fatty acids

PLFAs are stable components of the cell wall of most microorganisms (Winding *et al.* 2005). Individual PLFAs are specific for specific subgroups of microorganisms, i.e. Gram-negative or Gram-positive bacteria, methanotrophic bacteria, fungi, myccorriza, and *Actinomycetes* (Zelles 1999).

Phospholipid fatty acid analysis can be used to detect changes in the structure of microbial communities (Wilkinson *et al.* 2002, Winding *et al.* 2005), and although several fatty acids are specific to microbial main taxa, the PLFA technique is broad-scaled, picturing perturbations in the total microbial community structure after changes in growth conditions (Zelles 1999).

Winding *et al.* (2005) noted that, PLFA analysis has been also used to detect a pollution gradient in soil and found to be more discriminatory than Biolog measuerements for characterizing soil microbial communities.

The PLFA method has the advantage over DNA fingerprinting techniques that analysis is performed on microbial lipids extracted directly from the soil and is therefore considered a measurement of the *in situ* situation in the soil (Johansen and Olsson 2005).

The procedure for extraction, purification, and derivatization of fatty acids was described in detail by Thirup *et al*. (2003). According this method, mix of chloroform (7.6 ml) and methanol (15 ml) should be applied and the Teflon centrifuge tubes should be capped, agitated for 10 min and left overnight. After centrifugation (2500 x g for 10 min) the supernatant should be transferred to glass tubes. The pellet are subjected to an additional washing/centrifugation step and the two supernatants are pooled and dried under streaming N_2.

For purification and derivatisation of the polar lipid fraction, the gas chromatography (GC) equipment should be used, whereas an identification of fatty acids should be performed with use of the fatty acid nomenclature of Tunlid and White (1992).

Some authors (Federle 1986, Wilkinson 1988, O'Leary and Wilkinson 1988), indicated that, the selected PLFAs are the following: Gram-positive bacteria (PLFAs i15:0, a15:0, i16:0, i17:0, a17:0), Gram-negative bacteria (18:1-7,cy17:0, cy19:0), fungi (18:2-6).

6.3.9. Fluorescent *in situ* hybridization

FISH is a cytogenetic method developed by Christoph Lengauer, and is commonly used for detection and localization the presence or absence of specific DNA sequences on chromosomes (Wagner *et al.* 2003).

The specificity of FISH technique is that it uses fluorescent probes, that bind to only those parts of the chromosome with which they show a high degree of sequence similarity (Pernthaler *et al.* 2002).

In other words, FISH is a method in which single-stranded nucleic acids (usually DNA, but RNA may also be used) are permitted to interact, so that complexes, or hybrids, are formed by molecules with sufficiently similar, complementary sequences.

The method comprises of three basic steps:

- fixation of a specimen on a microscope slide or in a suspension,
- hybridization of labeled probe to homologous fragments of genomic DNA, and
- enzymatic detection of the tagged target hybrids.

In order to find out and confirm where the fluorescent probe is bound to the chromosomes the fluorescent microscopy is necessary.

O'Connor (2008) demonstrated that:

a). the basic elements of FISH technique are an isolated DNA probe and a target sequence,

b). DNA probes, before hybridization must be labeled according to the two the most common labeling strategies:

- indirect labeling – when probes are labeled with modified nucleotides, contained a hapten (small molecule that can elicit an immune response only when attached to a large carrier such as a protein),
- direct labeling – when nucleotides have been directly modified to contain a fluorophore.

c). denaturation of labeled probes and DNA target,

d). annealing of complementary DNA sequences,

e). in case of indirect labeling the probe, an extra one step is required for visualization of the non fluorescent hapten (enzymatic or immunological detection system). Whereas FISH is faster with directly labeled probes, indirect labeling offers the advantage of signal amplification by using several layers of antibodies, and it might therefore produce a signal that is brighter compared with background levels (O'Connor 2008).

Pernthaler *et al*. (2002) indicated that during hybridization the samples are incubated at elevated temperature in an airtight vessel saturated with water and formamide vapours of additional hybridization buffer to avoid concentration effects due to evaporation. The washing step is performed at a slightly higher temperature and serves mainly to rinse off excess probe molecules at conditions that prevent unspecific binding.

FISH technique is often used for finding specific features in DNA for use in genetic counseling, medicine, and species identification. FISH can also be used to detect and localize specific mRNAs within tissue samples. In this context, it can help define the spatial-temporal patterns of gene expression within cells and tissues.

Furthermore, FISH is widely used in the field of microbial ecology, to identify microorganisms (Wagner *et al.* 2003). Preparing DNA probes for one species and performing FISH with this probe allows one to visualize the distribution of this specific species in the soil samples. Preparing probes (in two different colors) for two species allows to visualize/study co-localization of these two species (Pernthaler *et al.* 2002).

Typical results of FISH experiment, in which an isolated DNA sequence was hybridized by NSO1225 probe to confirm in the *Eutric Cambisol* and *Haplic Phaezoem* sample the presence of AOB bacteria group (Wolińska 2009) are presented in Fig. 8, whereas hybridization with probes: Mg705 and Mg84 characterizing for methanothrophic bacteria Type I and Type II (Pytlak 2011) demonstrated Figure 9.

During recent years FISH has been successfully applied in freshwater, coastal and offshore marine planktonic habitats, as well as in coastal sediments (Pernthaler *et al.* 2002).

Group-specific probes for different subclasses of the *Proteobacteria* have, i.e., been utilized to study the composition of lake snow (Weiss *et al.* 1996), whereas blooms of members of the *Cytophaga* and *Flavobacterium* cluster have been described at the Antarctic marginal ice zone during a *Phaeocystis* bloom (Simon *et al.* 1999). Archaeal seasonal abundances and vertical distributions in Antarctic

coastal waters were followed by FISH with oligonucleotide and polynucleotide probes (Murray *et al.* 1998). Other applications of FISH are too numerous to be listed here. An overview of early applications can be found in Amann *et al*. (1995).

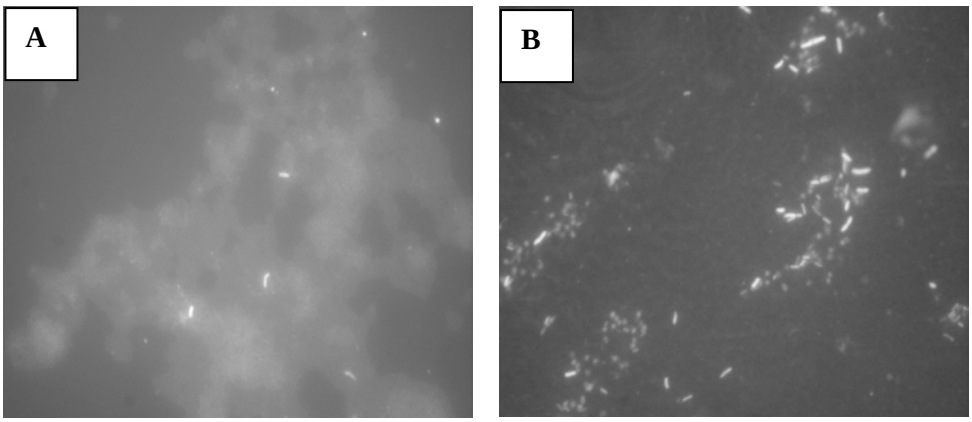

Fig. 8. AOB group of bacteria presented in the *Eutric Cambisol* (A) and *Haplic Phaeozem* (B) sample. FISH with NSO1225 probe (Wolińska 2009)

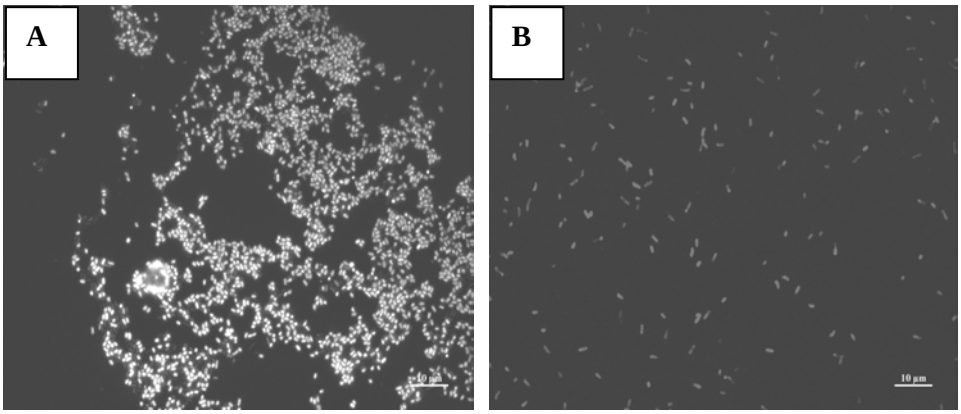

Fig. 9. Type I (A) and *Type II* (B) of methanothropic bacteria in a coal mine rocks. FISH with Mg705 and Mg84 probes (Typ I) and Ma450 probe (Typ II) (Pytlak 2011)

7. PERSPECTIVES

One of the most relevant environments, where there is still much to discover, is soil. Soil appears to be a major reservoir of microbial genetic diversity and is considered as a complex environment.

In the 20th century, the view of soil microbial ecology shifted from being described as a “clear picture” to diversity “beyond practical calculation”.

We have shown that neither view is entirely correct, however that estimate of bacterial richness is possible using a set of molecular techniques that have a reasonable biological potential.

Traditionally microbiologists have characterized soil microorganisms by studying either individual strains that could be cultivated in laboratory conditions. However, this approach provide rather little information on soil microbial communities since the vast majority of soil inhabitants are uncultured or at least, very difficult to culture.

Deoxyribonucleic acid is ubiquitous in the soil environment due to the fact that an extracellular DNA cycle in soil is not a closed system. It is rather continually replenished process caused by DNA releasing into soil by living and decomposing organisms. In addition, DNA is removed from the cycle through extracellular degradation or uptake by organisms to be reincorporated into new DNA (salvage and transformation) or other molecules (degradation).

Microbiology has relied long on diverse methods for DNA analysis, with improved technologies, stimulated by recent triumphs in the field, and attraction of diverse scientists to identify new problems and solve old ones. Molecular techniques are a new approach to provide the tools to balance the abundance of knowledge attained from culturing with an understanding of the uncultured majority of microbial life.

We should however realize, that current knowledge of soil microbial diversity is still limited by the inability to study all soil microorganisms. Most of them, including many model microbes are difficult or even impossible to cultivate. More and more we have to realize that the cultivation of microorganisms from the environment is a simplification of a reality leading to a wrong interpretation of the ecosystem.

Some of soil DNA isolation methods give the ability of obtaining most part of microbial DNA from the sample. What is more, the analysis of microbial community structures, using DNA technologies can profile whole community without unreliable cultivation steps and detect microbes which could not be detected using

traditional culturing methods. Even though, methods to study diversity (numerical, taxonomic, structural) are improving for both bacteria and fungi, however, there is still not a clear association between diversity and function.

Although molecular methods have the advantage of obtaining information about uncultivable organisms, they also have limitations that cannot be ignored. That is why it is still difficult to state whether one technique of studying soil microbial diversity is proper and better, than another. In order to obtain the broadest picture and the most information, the best way to study soil microbial diversity would be to use a variety of tests with different endpoints and degrees of resolution.

Thanks to combining methods of different fields, it may be possible to achieve more accurate information about the DNA, which has been released into the soil environment, and which can be helpful in receiving information about particular parts of soil DNA cycle.

Ongoing discussion of the relevance of different molecular techniques is inevitable and healthy for the scientific development of this methods. An experience gained from both, mistakes and successes, should be published and shared for being helpful in better understanding of the microbial communities and their importance for soil quality.

Moreover, in order to understand the complexity of interacting soils biological, chemical, and physical factors, botanists, microbiologists, pedologists as ecologists should strongly cooperate together.

We also should believe that as new molecular techniques are developed, modified and improved, our level of soil microbial world understanding will also quickly increase and our knowledge expands.

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9. SUMMARY

Deoxyribonucleic acid is a ubiquitous compound of the soil environment, constituting genetic information indispensable for soil microorganisms biodiversity determination.

Year of 2010 has been announced by UN as International Year of Biodiversity, in order to paying an attention on one large section of global biodiversity for too long ignored: soil biodiversity.

However, knowledge about soil microbial biodiversity is still limited, most of all by the fact that only 1% of soil microorganisms population could be isolated by use of traditional laboratory methods. Soil bacteria consist the huge quantity of genetic information, which constantly remained inaccessible for traditional microbiological tests. That is why, progressive techniques of molecular biology are more and more exploited at investigations of bacterial population in the soil environment.

An isolation and DNA analysis complete with more and more precise molecular techniques (i.e. PCR with its variety, CLPP, PLFA, DGGE, FISH) are nowadays the basic and how important explorative tools, approximating us to closer recognition of life environment and biodiversity of soil organisms.

In the current work the most popular molecular methods useful in the soil DNA determination and leading to microbial identification are presented. Choice of the proper molecular method should be anyhow adjusted to the problem, which may be solved: detection, identification, differentiation, taxonomic investigations. Technical aspects are also of great importance: analysis difficulties, experience, research background, costs of method applied.

Description of both advantages as disadvantages of the most popular analytical methods should be helpful for researchers standing before decision about selection of the proper technique for soil DNA analysis.

Keywords: soil, DNA, molecular techniques, PCR, soil microorganisms

10. STRESZCZENIE

SPOSOBY MOLEKULARNYCH ANALIZ KWASÓW NUKLEINOWYCH W BADANIACH ŚRODOWISKA GLEBOWEGO

Kwas dezoksyrybonukleinowy jest powszechnie obecny w środowisku glebowym, stanowiąc informację genetyczną niezbędną do określenia bioróżnorodności środowiska.

Rok 2010 został ogłoszony przez ONZ Rokiem Różnorodności Biologicznej, tym samym zwracając uwagę, iż nie można już dłużej pomijać tak ważnego, lecz często zapominanego aspektu różnorodności biologicznej – bioróżnorodności gleby.

Wiedza dotycząca bioróżnorodności mikroorganizmów jest ciągle ograniczana, głównie przez fakt, iż tylko około 1% populacji glebowych mikroorganizmmów może być izolowana przy użyciu tradycyjnych metod laboratoryjnych. Bakterie glebowe zawierają zaś ogromną ilość informacji genetycznej, która dotąd była niedostępna klasycznymi metodami mikrobiologgicznymi. Stąd też nowoczesne techniki biologii molekularnej są coraz częściej wykorzystywane do badania populacji bakterii w środowisku glebowym.

To właśnie izolacja i analiza DNA łącznie z coraz bardziej precyzyjnymi technikami molekularnymi (np. PCR i jego odmiany, CLPP, PLFA, DGGE, FISH) stanowią obecnie podstawowe i jakże ważne narzędzia badawcze, przybliżające nas do dokładniejszego poznania środowiska życia i różnorodności drobnoustrojów.

W pracy przedstawiono przegląd najbardziej popularnych metod molekularnych stosowanych w oznaczeniach glebowego DNA, celem których jest pozyskanie produktu jak najlepszej jakości, umożliwiającego identyfikację mikroorganizmów glebowych. Wybór metody molekularnej należy jednakże dostosować do problemu, który chcemy rozwiązać: wykrywanie, identyfikacja, różnicowanie, badania taksonomiczne. Nie bez znaczenia są także aspekty techniczne: stopień trudności analizy, doświadczenie, zaplecze badawcze, koszty stosowanej metody.

Prezentacja zarówno zalet jak i wad, a także najbardziej powszechnych zastosowań różnych metod analitycznych powinna być pomocna i stanowić ukierunkowanie dla badaczy stojących przed wyborem właściwej techniki analizy glebowego DNA.

Słowa kluczowe: gleba, DNA, techniki molekularne, PCR, mikroorganizmy glebowe

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Section and sub-section titles should be numbered with Arabic numerals and identified as follows:

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Tables: All descriptions of tables (titles and headings) should be in type size 9. Full wording should be used in table titles, i.e. **Table 1.,** and the word "table" should be in bold type; no dot should be used at the end of table title; justification on both sides, e.g.:

Table 1. Composition of batters of finely comminuted sausages of the wiener type

In the text of the paper full wording should be used, e.g. Table l, and when quoting, an abbreviated form should be put in brackets – (Tab. 1). Text in table headings should begin with a capital letter. If explanations are added below the table, they should end with a dot. Tables should be composed without side border lines and internal division lines. Only thin horizontal lines defining the table beginning and end (from above and below) are to be used, as well as lines underlining the headings.

Figures: In figure captions the abbreviated form should be used - **Fig. 1.**, and no dot should be placed at the end of the caption. In the text of the paper the full word „Figure" should be used, and when quoting, the abbreviated form in brackets (Fig. 1). Figure captions are to be written with justification on both sides, e.g.

Fig. 1. Relation between Fe(II) ions (mg dm^{-3}) activated in the loess soil and the experimental time at two temperatures

In axis description in figures, the following principle is used – the description is begun with a capital letter, and units used are given in brackets, e.g. Moisture (%)

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Italics should be used, both in the text and in formulae, to highlight the following:

– symbols of physical values;
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– Latin names.

Straight characters are used for:

– index exponents of powers and numerical upper and lower fractions (2^2, b^3, t_2, k_2);
– abbreviations of trigonometric and hyperelliptic functions (cos, tg), symbols of vector operators (grad, div), root and integer signs and constant functional symbols (d, f, π, Σ, const, exp), symbols of units of measurement (Ω, μm), symbols of measurement units in lower indexes (h_{m}), symbols of chemical elements (Cu, k_{Fe}), symbols of physical constants (Re – Reynold's number), designation of machine and apparatus types, letters in figure numbering (Fig. 15a), all brackets.

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Example:

REFERENCES

Horabik J., 1994. Load distribution in a model bin as influenced by mechanical properties of wheat grain (in Polish). Acta Agrophysica, 1.

Jury W.A., Roth K., 1990. Transfer Function and Solute Movement through Soil: Theory and Applications. Birkhäser Verlag, Basel, Switzerland.

Ostrowski E.W., 1971. Evaluation of sugar beet mechanical properties (in Russian). Sacharnaja Promyszlennost, 1, 17-20.
Rampazzo N., Blum W.E.H., Strauss P., Čurlik J., 1993. Structure assessment of two agricultural soil of Lower Austria. Int. Agrophysics, 7, 47-59.
Tardieu F., 1991. Spatial arrangement of maize roots in the field. In: Plant Roots and Their Environment (Eds B.L. Mc Michael, H. Persson). Elsevier, Amsterdam, 506-514.

SI system is compulsory throughout. Units should be given as powers in round brackets – (m s^{-1})

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Tabela 1. Charakterystyka badanych odmian
Table 1. Characteristics ……

W tekście używamy pełnego zapisu np. tabela 1, lub w tabeli 1, a cytując zapisujemy w nawiasie – (tab. 1).

Tekst w nagłówkach tabeli należy rozpoczynać z dużej litery. Jeżeli pod tabelą znajdują się objaśnienia należy zakończyć je kropką. Tabele należy składać bez linii bocznych i wewnętrznych. Powinny one mieć tylko cienkie linie poziome zamykające tabelę od góry i od dołu oraz podkreślające nagłówek.

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Rys. 1. Zależność ilości jonów Fe(II) uruchamianych z gleby lessowej ($mg \cdot dm^{-3}$) od czasu trwania doświadczenia w dwóch temperaturach

Fig. 1. Relation between Fe(II) ions (mg dm^{-3}) activated in the loess soil and the experimental time at two temperatures

W opisach osi rysunków stosujemy następująca zasadę: zaczynamy dużą literą i podajemy jednostkę w nawiasie okrągłym, np. **Wilgotność – Moisture (%).** Jeśli opis jest długi zapisujemy wersję polską w jednej linijce, angielską w drugiej, a po niej jednostkę, np.

Udział ziaren uszkodzonych i zdolność kiełkowania
Share of damaged grains and germination capacity (%)

Wzory: należy zapisać czcionką nr 11, wyrównywanie centralne. Odstęp nad i pod wzorem powinien wynosić 0,5 cm. Wzory powinny być ponumerowane, a numery należy umieścić w nawiasach okrągłych przy prawym marginesie.

Kursywą należy wyróżnić zarówno w tekście jak i we wzorach:
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- nazwy łacińskie.

Prostym pismem składa się:
- cyfrowe wykładniki potęg oraz cyfrowe frakcje górne i dolne (2^2, b^3, t_2, k_2);
- skróty funkcji trygonometrycznych i hiperbolicznych (cos, tg), symbole operatorów wektorowych (grad, div), znaki pierwiastka i całki oraz stałe symbole funkcyjne (d, f, π, Σ, const, exp), symbole jednostek miary (Ω, μm), symbole jednostek miary w indeksach dolnych (h_m), symbole pierwiastków chemicznych (Cu, k_{Fe}), symbole stałych fizycznych (Re - liczba Reynoldsa), oznaczenia typów maszyn i przyrządów, litery przy numerach rysunków (Rys. 15a), wszelkie nawiasy.

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Horabik J., 1994. Wpływ właściwości mechanicznych ziarna pszenicy na rozkład obciążenia w zbiorniku. Acta Agrophysica, 1.

Jury W.A., Roth K., 1990. Transfer Function and Solute Movement through Soil: Theory and Applications. Birkhäser Verlag, Basel, Switzerland.

Ostrowski E.W., 1971. Opriedielienije mechaniczeskich swoistw sacharnoj swiokły. Sacharnaja Promyszlennost, 1, 17-20.

Rampazzo N., Blum W.E.H., Strauss P., Čurlik J., 1993. Structure assessment of two agricultural soil of Lower Austria. Int. Agrophysics, 7, 47-59.

Tardieu F., 1991. Spatial arrangement of maize roots in the field. In: Plant Roots and Their Environment (Eds B.L. McMichael, H. Persson). Elsevier, Amsterdam, 506-514.

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