Modern techniques used for biodiversity analysis in bacterial environmental communities

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Introduction

Research methods based on molecular biology techniques are very popular in modern environmental laboratories. They replace traditional microbiological methods which are based on morphological characteristics, pathogenicity, nutritional requirements or ecological niches [1]. These methods possess several disadvantages – it is impossible to isolate pure cultures and to cultivate some kinds of bacteria in artificial conditions e.g. – activated sludge bacteria, which take part in biological treatment of wastewater (nitrifying and denitrifying bacteria, PAO – polyphosphate accumulating organisms) are not cultivable in laboratory conditions. Moreover, they do not possess particular phenotype features, grow slowly and they are affected by the environmental factors [2]. In opposition to classical microbiological methods, molecular characteristics are easier and faster. Growth medium is not demanded, because probes used in the analysis are taken directly from the environment. These tools enable to study all types of bacteria, including nonculturable, under laboratory conditions [2]. Moreover, samples for analysis may be small, and results are repeatable. That is why molecular methods become more and more popular in technological and microbiological laboratories, and can be used to monitor activated sludge biocenosis in wastewater treatment plants and the other biological research systems. The most popular molecular biology tools for bacterial community analysis are: fluorescent *in situ* hybridization (FISH), flow cytometry (FCM) and denaturing gradient gel electrophoresis (DGGE).

Fluorescent *in situ* hybridization (FISH)

Fluorescent *in situ* hybridization is based on classical hybridization method. Stabile double – helix structures are constructed between single stranded molecular probe and single stranded complementary polynucleotide sequence. This method allows to find position of gene, sequence or molecular marker directly in RNA or DNA molecules. It is possible because of probe labeling. Formerly for labeling radioactive substances were used, but that generated problems with high sensitivity and resolution of images [3]. Consequently, other solutions were needed. Fluorescent labeling solved this problem successfully. Nowadays, there are a lot of molecular labels with different emission colors that allows the usage of several probes for one chromosome and the obtainment of difference single hybridization signals with fluorescence color [3]. Observation is possible while using fluorescent or confocal microscope. Sample is illuminated with a particular wavelength of light which excites the fluorescence of a particular fluorochrome. The most popular probes for FISH are polynucleotides, because it is possible to label them several times. There are three types of polynucleotide probes: dsDNA (double-stranded DNA), ssDNA (single-stranded DNA) and ssRNA (single-stranded RNA). ssRNA probe is probably the most stable of them [4]. In bacterial biocenosis biodiversity analysis, probes complementary to 16S rRNA and 23S rRNA ribosomal sequences are commonly use. A lot of research projects resulted in a vast sequence database [5÷7]. They include sequences of bacteria which we are able to culture in laboratory conditions as well as bacteria obtained from environmental samples [8÷9].

Hybridization degree of labeled probe and complementary sequence (so called duplex), depends on a variety of physical and chemical conditions (Tab. 1). The main are: temperature, pH, molecular probe length, sequence divergence, percentage G+C content, single and double-valent salt cations, and formamide concentration which denature nucleic acids [4]. Duplex stability is measured by melting temperature (T_m). It has been assumed that the higher T_m, the more stable duplex structure is [3]. Every duplex is characterized by different, specific to this sequence, melting temperature. In this temperature double-stranded molecule separates into two single-stranded polynucleotides, which is an effect of hydrogen bonds breaking.

Table 1

Correlation between stability of probe-target duplexes and hybridization parameters [4]

Parameter	Duplex stability (depending on T_m)
temperature	decreasing when T_m grow
pH	without influence when ranged between 6 and 8
molecular probe length	-for DNA:DNA hybrids -> D=500/L -for DNA:RNA and RNA:RNA hybrids -> D=820/L D – T_m reduction (in °C), L – length of paired sequence in duplex
sequence divergence	1% mismatch effect in 0.5-1.4°C T_m decrease
% G+C	higher when the %G+C content increases
single-valent salt cations	higher when concentration of Li^+, Na^+, K^+, Cs^+, Rb^+ increases
double-valent salt cations	higher in correlation with concentration increase
formamide	-for DNA:DNA hybrids there is a linear correlation between T_m decrease and its concentration (0.6°C for 1%) -for DNA:RNA hybrids correlation between T_m decrease and formamide concentration is not linear (T_m decrease is lower in high formamide concentration conclusion: in higher formamide concentrations DNA:RNA duplexes are more stable than DNA:DNA duplexes

FISH technique possesses a big advantage – it does not influence the integrity and surrounding of bacterial chromosome [10]. The first step in procedure is to create molecular probe with sequence complementary to the searched one. It has to be specific to the target gene. Second step is its labeling with a fluorochrome – it is an important part of analysis because too high or too low labeling rate, improper probe purification from the excess of the reagents or probe fragmentation can cause the falsification of the experiment results.

Preparation of biological material for analyses consists of fixation of cells on glass slide and then, the fluorescently labeled molecular probe is applied onto the specimen. The reaction mixture is then denatured, mostly in high temperature or organic solvents (e.g. in

formamide). This procedure ensures preservation of the original structure of the chromosome [3]. Next stage is the hybridization, preparation rinsing and genetic material visualization. For this aim, the most commonly used DNA binding dye is DAPI (4,6-diamidino-2-phenylindole). It binds strongly to the double stranded DNA according to the principle of intercalation – insertion of neighboring base pairs. The preparation is ready to be observed under fluorescent or confocal microscope.

Basic procedure of fluorescent *in situ* hybridization is commonly used in bacteria biocenosis research. Also important in this kind of analysis are the modifications of FISH: RING-FISH, CARD-FISH, MAR-FISH and Clone-FISH. In RING (**r**ecognition of **in**dividual **g**enes in a single bacterial cell) – FISH the repeatedly labeled polynucleotide probes are used to detect single genes. For this type of FISH a "halo" signal, surrounding the cell is typical. Its origin is not entirely explained. Probably it is partly a result of probe-probe hybridization and linking with each other and partly probe hybridization with target sequence and other sequence inside the cell. The predisposition of polynucleotide probes to present this type of signal does not depend on their length, but on the ability to form the secondary structure and is strongly correlated with high concentration in specimen [4, 11].

CARD (**ca**talyzed **r**eported **d**eposition) – FISH plays an important role in quantitative analysis of microorganisms in aquatic ecosystems, so it is a valuable tool in activated sludge biocenosis research. Method is based on fluorescence signal amplification with using the tyramide (TSA – **t**yramide **s**ignal **a**mplification) and covalent binding the molecular probes with horseradish peroxidase. Modification is used because the intensity of original signal, which is the result of hybridization, is below the detection threshold. Appropriate analysis is often impossible due to high background fluorescence. High signal to background ratio is a reason why CARD – FISH is a useful method for identification cells with low ribosomal RNA content [12].

Clone – FISH modification is a method in which standard FISH procedure and gene cloning are combined. It is used when there is no possibility to prepare a molecular probe in the absence of the data about microorganism genome. To obtain molecular probe firstly the *in vivo* transcription of target gene coding 16S rRNA (in plasmid vector of *Escherichia coli)* is performed. Transcript is then used as a probe to search for the sequence and duplexes detection is performed by FISH [13, 14].

MAR (**m**icro**a**uto**r**adiography) – FISH, also known as MICRO – FISH is a combination of *in situ* hybridization and microautoradiography. In first step of this method is to label organic and inorganic substrates with radioisotope elements and incubated with the environmental sample. In the second stage genetic material in bacterial cells hybridizes with fluorescently labeled probe. Therefore it is possible to determine metabolic activity of analyzed biocenosis, substrate transport limitation and identification of collected substances on the single cell level. At the same time phylogenetic identification occurs. The method allows to find the composition and the particular bacteria function in the environmental sample [5, 13]. It has a wide application to study nitrifying and denitrifying bacteria, PAO (polyphosphate accumulating organisms), sulfur – oxidizing and sulphite – reducing bacteria, for identification xenobiotics – degrading microorganisms and to study the role of filamentous bacteria in activated sludge [5]. Schematic illustration of MAR-FISH is showed in Figure 1.

Recently FISH technique was used in research performed on the treatment of landfill leachates by nitrifying bacteria [15] and in analyzing the correlation between PAO microorganisms and denitrifiers in simultaneous denitrification and dephosphatation system in which organic carbon source varied [16]. The method was used also in the research about the competition of PAO and GAO (glycogen accumulating organisms) in AGS (aerobic granular sludge) reactor [17] and allowed to clear understanding of activated sludge flocs composition [18]. Chelliapan et al. [19] used FISH technique to identify bacteria in anaerobic reactor in which pharmaceutical wastewater was treated. Results showed, that dominant organisms in the system are methanogenic *Archaea* belonging to the *Methanosarcina* and *Methanosaeta* genera – they produce methane in the anaerobic respiration path. Degenaar et al. [20] applied the FISH method to estimate changes in activated sludge *Proteobacteria*, treating wastewater containing high concentrations of fatty substances. Phuong et al. [21] used fluorescent *in situ* hybridization in research of the role of *Acinetobacter* bacteria in activated sludge flocs forming, and the sedimentation process in settler tank.

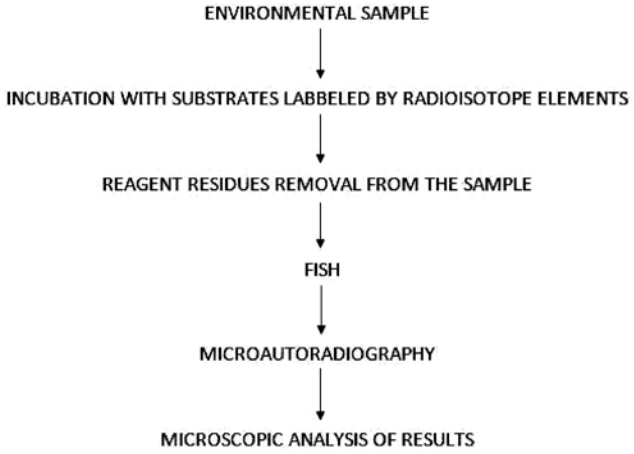

Fig. 1. The steps of MAR-FISH procedure

Flow cytometry (FCM)

Flow cytometry is an advanced analytical technique, based on measurement of chemical and physical parameters, such like fluorescence, bending and scattering of light by microorganisms. FCM allows to multiparametrical, quantitative and qualitative characterization of a single cells in a precise, repeatable and fast way [22]. Analysis is performed in flow cytometer connected to the computer. This device is a complex measuring apparatus in which hydraulic, optic and electronic systems can be distinguished. Function of hydraulic system is to deliver a suspension of analyzed cells to the measuring chamber. The suspension is surrounded with protective isotonic fluid when it passes through the chamber, flows evenly so cells agglomeration does not occur. The construction of the cytometer provides a laminar flow of cells and their low concentrations, so that they can pass through the channel separately. In a measuring chamber the cells are treated with the polarized light beam, perpendicular to the direction of the flow. During the flow cells are crossing the beam and emit fluorescence signals, which are then received by detectors: forward angle light scatter (FS), side scatter (SS) and fluorescence detectors. Signals are amplified, digitally processed and visualized electronically on a computer screen [23÷24].

Bending and scattering of light occurs in different directions:

- forward scattering – emitted light bends on the edges of cells in the same direction as light beams, so that it is possible to mark the size of cells. Detector FS is used
- side scattering – emitted light bends at right angle on cellular wall, cellular membrane or intracellular structures, enabling the detection of grains. Detector SS is used.

In case of the environmental samples, cells are additionally marked by fluorescent dyes (e.g. DAPI) or treated by labeled antibodies or polynucleotides. The fluorescence intensity of received signal is proportional to the amount of the dye. It is also possible to use other tracers or chemical compounds which allow to make broader spectrum of the analysis [25÷27].

Flow cytometry has been recently adapted as a research tool in biological composition of activated sludge biocenosis study (Tab.2). Small cells dimension and the fact, that they are alive in the suspension enabled to adjust this method for this kind of research.

Beyond the multi-dimensional statistical analysis to evaluate the structural parameters of cell, this technique allows also to detect the trace subpopulations. In cytometers additionally equipped with the sorter, sorting the cells in an electric field is also possible [28].

Table 2

Cell detection methods of bacterial cell populations using flow cytometry [44]

Physiological state of cells	Intact cells			dead cells
	metabolically active cells			
	cells capable of reproducing			
Criteria	cell division	metabolic activity	cell membrane integrity	cell membrane integrity
Detection method	cells counting cells 'tracking'	enzymatic activity membrane potential	selective membrane permeability	the investigations of anomalities in nutrients absorption

Recently flow cytometry has been used in studies of the changes dynamics in bacterial populations of activated sludge, bacteria in raw wastewater flowing into the treatment plant and on the effluent. The relationships between the amount of alive and dead cells in relation to the purification step and their quantitative ratio on all stages were analyzed [29]. Technique was also used in activated sludge analyzing during the excess sludge reduction mechanisms. It allowed visualizing and estimating the number of cells that are damaged or permeabilized during this process and in the consequence to determine the effectiveness of the treatment [30]. In research of Muela et al. [31] flow cytometry was used as a tool for fast analysis of inflowing and outflowing wastewater quality. By examining the biological environment of samples it was found that microbial indicators are better physico-chemical changes indicators than standard measurements of COD, BOD, pH or conductivity. It is associated with a higher sensitivity for changes in wastewater composition. Researchers have proposed measuring the amount of active bacterial cells as a standard indicator showing the condition of biological treatment plant and the flow cytometry method, as a quick and useful tool to determine this.

Denaturing gradient gel electrophoresis (DGGE)

Denaturing/**t**emperature **g**radient **g**el **e**lectrophoresis (DGGE/TGGE) is a technique that uses differences in melting temperatures (T_m) of double stranded DNA. Denaturing factor may by a chemical compound, such as urea (DGGE) or physical, such as temperature (TGGE – **t**emperature gradient). Electrophoresis is carried out in polyacrylamide gel containing an increasing gradient of denaturant. The gel wells nucleic acids fragments are set, previously amplified by PCR (polymerase chain reaction). Fragments differ in the sequence, but their length is the same. During the electrophoresis the hydrogen bonds are broken and partially single-stranded molecules with reduced electrophoretic mobility (retardation) are formed. This provides a separation of DNA fragments which have the same gel mobility in double-stranded forms in non-denaturing environment, but different melting properties (based on the sequence difference) [32]. In order to prevent complete separation of strands DNA fragments are amplified by PCR before electrophoresis with the primers pair in which one of them possesses GC clamp. It is a fragment composed of 30 – 40 GC base pairs, characterized by high melting temperature, so that even at high concentrations of denaturant separation of PCR products does not occur [33]. DGGE electrophoresis can differentiate nucleic acid fragments according to even one base difference. In result the fingerprints (DNA profiles) of samples are obtained. This allows to determine changes of biocenosis composition during time, so to determine biodiversity. It is also possible to isolate and excise the DNA bands from the gel and sequence them in order to the bacterial identification the scheme of DGGE gel after electrophoresis is showed in Figure 2.

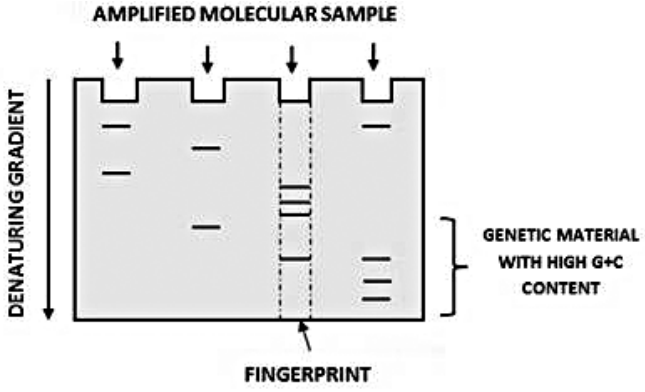

Fig. 2. Results of DGGE electrophoresis – fingerprints in polyacrylamide gel

DGGE method is repeatable, fast and relatively cheap. These features are related to its popularity in the molecular analysis of environmental samples [34]. DGGE was used to the study of bacteria group like AOB (ammonia oxidizing bacteria), NOB (nitrite oxidizing bacteria) and PAO. The tool allowed separating the most common groups in activated sludge bacteria and categorizing them into relevant subclasses, according to the genetic relatedness [35]. Using DGGE has also been found that the dominant role in the biological wastewater treatment plays bacteria from β-subclass of *Proteobacteria* [35]. Research performed on AOB bacteria confirmed this information. AOB was separated into two monophyletic subgroups (a group including all descendants of a common ancestor) related with *Proteobacteria*. It is γ-subclass, in which *Nitrosococcus oceani* is and β-subclass, including bacteria from *Nitrosomonas*, *Nitrosospira*, *Nitrosolobulus* and *Nitrosovibrio* genera [36]. Phylogenetic research about PAO organisms provided information about their classification as groups: α-*Proteobacteria*, β-*Proteobacteria* and *Actinobacteria* [37].

In recent years DGGE was used in bacteria biodiversity research of reactor in which ozonation was used to minimize the presence of the excess sludge. Analysis showed, that the ozonation process does not adversely effect on the biological environment of the reactor, moreover – biodiversity of ozonated reactor was higher than the one of the reactor not ozonated [38]. Also the effect of high temperature influence on the bacterial populations changes in the technology systems used for the same purpose as ozonation was studied. It was demonstrated that the treatment of sludge in temperature 90°C during 9 weeks, has changed the biodiversity by 59% in comparison with the control sample. Activated sludge lost the ability to nitrification, while the new bands in fingerprint appeared, probably derived from thermophilic bacteria [39] PCR-DGGE technique also showed the negative impact of carcinogenic tetrahydrofuran which was present in the effluent, on the biological environment of the reactors. Results showed, that it is necessary to search for the other solutions for this compound degradation before introduction the contaminated wastewater to the biological treatment plant [40]. Chang et al. [41] studied the influence of the other chemical compound – o-nitrobenzaldehyde, presents in industrial wastewater, on the SBR reactor biocenosis. Hersham et al. [42] used denaturing gradient gel

electrophoresis to study the biological structure of activated sludge in two research systems – in standard A2O system, and in reversed system, in which anoxic reactor was the first in technological system. In both systems, during the experiment period, problems with sludge bulking were encountered. DGGE allowed showing the difference in the composition of the two studied activated sludge systems, and determining that the probable cause of problems was the presence of filamentous bacteria *Microthrix parvicella* and *Nostocoida limicola*. Conclusions were pointed by the comparison of the fingerprints and the change of Mohlmann Index (sludge volume index – SVI). Bin et al. [43] used DGGE technique to study bacterial biodiversity of activated sludge granules in aerobic – anaerobic SBR system. It was showed that large granules are characterized by higher diversity of AOB bacteria than the smaller, moreover – that in large granules the inhibition of NOB bacteria growth occurred.

Summary

Molecular tools allow to study bacterial diversity in the environment, the area which the research were previously limited by the classical microbiological methods disadvantages. For this reason they become more and more popular in microbiological and technological laboratories. They allow studying both, total biocenosis and a single microorganism cell. Due to the fact that the taxonomic identification (the determination of microorganisms linkeage to the species), as well as the biodiversity of biota in the biocenosis are very important factors in activated sludge research – molecular techniques have a strong impact on the development of the other science fields, such as environmental biotechnology and environmental engineering. It is worth mentioning, that using methods which results are complimentary to each other enable to obtain total bacterial activated sludge biocenosis (and the other bacterial bocenoses) picture, together with these methods disadvantage minimization. Nonetheless, the choice of the right set of methods depends mainly on the study aim.

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Polska chemia dla poprawy bezpieczeństwa

17 października 2013 r. w Tarnowie, Spółki Grupy Azoty wraz z innymi polskimi firmami chemicznymi podpisały deklarację dotyczącą poprawy bezpieczeństwa pracy, ochrony przeciwpożarowej i ochrony środowiska w przemyśle chemicznym, w której zapisano m.in., że *„Celem zawieranego porozumienia jest wypracowanie modelu współpracy w obszarze bezpieczeństwa procesowego dla zmniejszenia zagrożeń mogących skutkować poważnymi awariami przemysłowymi. Sygnatariusze zobowiązują się do wymiany doświadczeń z obszaru bezpieczeństwa procesowego i pracy oraz prowadzenia działań prewencyjnych „Zero wypadków i awarii"*.

Deklaracja ma charakter otwarty stwarzając tym samym możliwość ciągłej wymiany doświadczeń z firmami spoza Grupy Kapitałowej. *(em)*

(Informacja prasowa Grupy Azoty, 17 października 2013 r.)

Międzynarodowa Wystawa i Konferencja NAFTA I GAZ

Podczas Międzynarodowej Wystawy i Konferencji NAFTA I GAZ 2013 (18–19 września 2013 r. w Warszawie) ICSO „Blachownia" został uhonorowany, wraz z Centrum Naukowo Badawczym Ochrony Przeciwpożarowej oraz P.P.H. Chemkonfekt, wyróżnieniem za „Środek odtłuszczający do usuwania substancji ropopochodnych z powierzchni przemysłowych". *(kk)*

(http://www.icso.com.pl/, 4.10.2013)

Związki Biologicznie Czynne – Aktywność, Struktura, Synteza

W dniach 4–6 października 2013 roku odbyła się w Białymstoku III Konferencja „Związki Biologicznie Czynne – Aktywność, Struktura, Synteza", zorganizowana przez Białostocki Oddział PTChem i Instytut Chemii Uniwersytetu w Białymstoku. Uczestniczyło w niej ok. 60 osób z Warszawy, Łodzi, Krakowa i Gdańska, a także z uczelni białostockich. Tematyka konferencji obejmowała zagadnienia związane z syntezą i badaniami strukturalnymi związków biologicznie czynnych w odniesieniu do szerokiego spektrum ich działania. W programie znalazły się wykłady zaproszonych prelegentów takich jak: prof. Jadwiga Frelek (ICHO PAN Warszawa), prof. Marek Potrzebowski (CBMiM PAN w Łodzi), prof. Ryszard Andruszkiewicz (Politechnika Gdańska), prof. Jacek Młynarski (UJ), prof. Jacek Morzycki (UwB). Ponadto prezentowane były komunikaty ustne i postery. Podobnie jak dwie poprzednie, konferencja miała charakter kameralny, co stwarzało możliwość ciekawszych dyskusji zarówno podczas sesji jak i poza nimi.

W ocenie organizatorów i uczestników konferencja spełniła założone cele takie jak nawiązywanie i podtrzymywanie kontaktów między różnymi ośrodkami prowadzącymi badania nad związkami biologicznie czynnymi. Ważny był także udział młodych pracowników nauki, którzy mieli okazję do wysłuchania bardzo ciekawych wykładów, a także do zaprezentowania i przedyskutowania własnych osiągnięć. Następna konferencja planowana jest za dwa lata. *(em)*

(Informacja prasowa Organizatorów Konferencji, 19 października 2013 r.

dokończenie na stronie 1133