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Detection of Sulfonamide Resistance Genes *via in situ* PCR-FISH

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Abstract

Due to the rising use of antibiotics and as a consequence of their concentration in the environment an increasing number of antibiotic resistant bacteria is observed. The phenomenon has a hazardous impact on human and animal life. Sulfamethoxazole is one of the sulfonamides commonly detected in surface waters and soil. The aim of the study was to detect sulfamethoxazole resistance genes in activated sludge biocenosis by use of *in situ* PCR and/or hybridization. So far no FISH probes for the detection of SMX resistance genes have been described in the literature. We have tested common PCR primers used for SMX resistance genes detection as FISH probes as well as a combination of *in situ* PCR and FISH. Despite the presence of SMX resistance genes in activated sludge confirmed via traditional PCR, the detection of the genes via microscopic visualization failed.

Key words: Sulfamethoxazole resistance gene, activated sludge, FISH, *in situ* PCR

Introduction

The spread of drug resistance poses a significant threat to health. Diseases caused by antibiotic-resistant bacteria are difficult to treat, last longer and pose the risk of death. In addition, they also contribute to increased costs of treatment, because often their eradication requires the use of alternative and more expensive drugs. WHO (2013) stresses that antimicrobial resistance (AMR) is a problem of a global concern and among several factors that accelerate the emergence and spread of AMR quotes weak or absent antimicrobial resistance surveillance and monitoring systems. Antimicrobial resistance transfer occurs the most often among environmental bacteria exposed to unmetabolised pharmaceuticals or their metabolites. Wastewater treatment plants are known to be a great AMR reservoir and resistance transfer place. It has been proved (Silva *et al.*, 2007) that there are more antibiotic resistant bacteria in treated wastewater than in raw wastewater. There is a need to develop methods suitable for fast and inexpensive detection of resistance genes in environmental samples enabling to follow the resistance transfer and its favoring factors.

There is a lack of a simple method for the tracking of genes to assess conditions that induce and maintain drug resistance phenomena at the level of single cell or particular population, especially in environmental samples. The aim of the study was to detect the selected resistance genes in activated sludge *in situ* and to visualize them via microscopic observations to recognize which group of activated sludge bacteria carries more resistance genes. Two ways have been chosen to reach the aim: fluorescence *in situ* hybridization (FISH) using *PCR* primer-based oligonuclotides probes and hybrid techniques *in situ* PCR and FISH, as *in situ* methods enable analysis directly in the environmental matrix, without the necessity of nucleic acids or cell extraction. *In situ* PCR has been used for the visualization of specific genes (Hodson *et al.*, 1995; Hoshino *et al.*, 2003) while FISH is commonly used mainly for the detection and visualization of species or genera specific sequences in mixed environmental populations (Daims *et al.*, 2005). Moreover, molecular methods such as PCR and FISH enable analysis without cell cultivation in contradiction to other techniques frequently used for drug resistance susceptibility assessment.

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Sulfonamide resistance genes were chosen to verify the suitability of this hybrid method as the genes are frequently present in activated sludge. Sulfonamides (*e.g.* sulfamethoxazole, widely used throughout the world in medicine and veterinary in treatment and protection against infections) are known inhibitors of bacterial dihydropteroate synthase (DHPS). In bacteria, the enzyme DHPS participates in the folic acid pathway. Deficiency of folic acid prevents production of purines necessary for the synthesis of RNA (ribonucleic acid) and DNA (deoxyribonucleic acid), which leads to inhibition of bacterial cell growth (Brunton, 2007; Skold, 2001).

Bacterial resistance to sulfonamides occurs through mutations in the chromosomal DHPS gene (*folP*) or through the acquisition of an alternative DHPS gene (*sul*), whose product has a low affinity for sulfonamides. *Sul* genes are the most common mechanism of sulfonamide resistance (Skold, 2000; Perreten and Boerlin, 2003). There are three *sul* genes encoding resistance to sulfonamides found Gram-negative bacteria plasmids: *sul1*, *sul2* and *sul3* (Grape *et al.*, 2003; Antunes *et al.*, 2005). The *sul*1 gene is mostly found linked to other resistance genes in class 1 integrons. The *sul*2 is usually located on small plasmids of the *inc*Q group, or on small plasmids represented by pBP1 (Antunes *et al.*, 2005). The sul3 gene specifies a 263-amino-acid protein similar to a dihydropteroate synthase encoded by the 54-kb conjugative plasmid pVP440 from Escherichia coli (Perreten and Boerlin, 2003). Resistance to sulfonamides is generally attributed to the presence of *sul1* and *sul2* genes.

Experimantal

Materials and Methods

Activated sludge sampling. Samples of activated sludge were collected from municipal wastewater "Zabrze Śródmieście" located in Zabrze, Poland. Activated sludge samples were taken from nitrification chamber and settling tank in the wintertime and summertime (4 samples). A sample of activated sludge was treated differently for various methods. That dedicated for PCR was frozen and stored at –20°C. That used for FISH was immediately fixed with 4% paraformaldehyde (incubation time of 90 minutes, 4°C) and after washing step with 1xPBS stored at –20°C.

Detection of SMX resistance genes by PCR. Total genomic DNA was isolated from sludge samples with the Fast DNA spin kit for soil (MPBiomedicals). Presence of DNA was determined with agarose (0.8% in 1xTBE) electrophoresis. Fragments of the *sul1* and *sul2* gene coding the sulfamethoxazole resistance were amplified by PCR. PCR reactions contained 5 μM primers, 1X PCR buffer, 20 μM dNTPs, 1 mM $MgCl_2$, and 1,5 U of GoTAQ Flexi DNA polymerase (Promega Corp., Madison, WI, U.S.A.) and were run in a C1000 Touch (BIO-RAD). Primer sequences for *sul1* and *sul2* amplification are presented in Table I. PCR was carried out under the following cycling regime: 5 min at 96°C; 1 min at 55°C, 3 min at 70°, 23 cycles each of 15 s at 96°C, 30 s at 55°C and 3 min 72°C. Final extension was carried out for 5 min at 72°C (Grape *et al.*, 2003). The quality of PCR products was determined visually on a 1 × TBE, 0.8% agarose gel.

Detection of SMX resistance genes by FISH. Activated sludge samples were fixed with 4% paraformaldehyde. The detection of SMX resistance genes was carried out using fluorescence *in situ* hybridization (FISH) with probes whose nucleotide sequence was based on the sequence of forward PCR primer used for detection of SMX resistance genes (Kadlec *et al.*, 2005). The 5'-end of the oligonucleotide was modified with a fluorescent dye – Cy3 in order to visualize the hybridization product. The probes were obtained from Biomers (Ulm, Germany). *In situ* hybridization was performed as described previously by Daims *et al.* (2005).

Since the optimal hybridization conditions (hybridization and wash buffer composition) for detection of

Table I
Oligonucleotide probes used in this study

Probe name	Method	Sequence (5'-3')	Reference
EUB 338 I	FISH	GCT GCC TCC CGT AGG AGT	Amann *et al.*, 1990
EUB 338 II	FISH	GCA GCC ACC CGT AGG TGT	Daims *et al.*, 1999
EUB 338 III	FISH	GCT GCC ACC CGT AGG TGT	Daims *et al.*, 1999
SUL1	FISH	CTA GGC ATG ATC TAA CCC TCG GTC	Kadlec *et al.*, 2005
SUL2	FISH	ACA GTT TCT CCG ATG GAG GCC	Kadlec *et al.*, 2005
*sul*1F	PCR	ATG GTG ACG GTG TTC GGC ATT CTG A	Grape *et al.*, 2003
*sul*1R	PCR	CTA GGC ATG ATC TAA CCC TCG GTC T	Grape *et al.*, 2003
*sul*2F	PCR	GAA TAA ATC GCT CAT CAT TTT CGG	Grape *et al.*, 2003
*sul*2R	PCR	CGA ATT CTT GCG GTT TCT TTC AGC	Grape *et al.*, 2003

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sulfamethoxazole resistance genes are not known, hybridization was carried out with a use of 10%, 20%, 30%, 40% and 50% formamide (FA) concentration in the hybridization buffer and adequate concentration of NaCl in washing buffer. Detection of the bacteria was made using a fluorescence microscope Motic400T. Before microscope observations, samples were embedded in Citifluor (Citifluor Ltd, United Kingdom) to reduce fluorochrome fading. Pictures were taken using camera Moticam Pro-285Acolor and Motic Images Plus 2.0 software.

Detection of SMX resistance genes by *in situ* PCR and FISH. Cell wall permeabilization and *in situ* PCR were carried out as described previously (Cheng and Hodson, 2001) with minor modifications. The main modification was the use of fluorescently labeled dUTP (dUTP-Cy3) together with standard mixture of dNTP in PCR in order to visualize the PCR product. Prior to cell wall permeabilization activated sludge was treated 1:1 with te lysozyme solution (2 mg/ml) for 15 min at room temperature. Further permeabilization was carried out optionally (see Table II) by treatment with DNase-free RNase at a final concentration of 5 μg/ml for 15 min at room temperature. Then, the samples were dehydrated sequentially in 50, 80 and 98% ethanol for 3 min each. At this stage the samples on the slides were ready for *in situ* PCR and FISH analysis.

Several series of tests were performed according to the results of a previously performed series and designed to verify the suitability of the method of *in situ* PCR-FISH for the identification of resistance genes in activated sludge bacteria. The experiment series are summarized in Table II.

In series A (a1-a2) *in situ* PCR methodology described by Cheng and Hodson (2001) was used to check the possibility of using two available DNA polymerases – Klenow fragment 3'-5' exo (Jena Bioscience) or GoTaq Flexi DNA polymerase (Jena Bioscience). Series B (b1-b3) sought to determine the effect of RNase on the FISH results (b1 and b2) and the impact of the PCR reaction (high temperature) on the feasibility of FISH (reaction b1 and b3). Series C (c1-c2) was performed to determine whether a fluorochrome originated from labeled dUTP can be separated from the labeled molecule during the PCR and bind nonspecifically to activated sludge. A series D (d1-d3) was designed to check the feasibility of traditional *in situ* PCR (without using the fluorescently-labeled dUTP) combined with FISH to detect functional genes in activated sludge bacteria.

In situ PCR was used for amplification of the *sul1* and *sul2* (Table III) gene in the bacterial cells. The PCR mixture contained PCR buffer 1x, 5 μM of each primer set (*sul1R*/*sul1F* and *sul2R*/*sul2F*), 200 μM of each of deoxynucleotide, and 20 μM of dUTP-Cy3, and 1 U of DNA Polymerase. PCR was performed with the using of following temperature profile: 5 min at 96°C; 1 min at 55°C, 3 min at 70°, 23 cycles each of 15 s at 96°C, 30 s at 55°C and 3 min 72°C. Final extension was carried out for 5 min at 72°C.

Table II
Characterization of experiment series performed to optimize *in situ* PCR-FISH

Series name	A		B			C		D		
Series goal	Polymerase suitability		Effect of high temperature and RNa-se on FISH			dUTP binding		Feasibility of *in situ* PCR-FISH		
Reaction name	a1	a2	b1	b2	b3	c1	c2	d1*	d2*	d3*
Sample pretreatment										
Fixation with PFA	+	+	+	+	+	+	+	+	+	+
Lysozyme treatment	+	+	+	+	+	+	+	+	+	+
RNa-se treatment	+	+	–	+	–	–	–	–	–	–
Ethanol dehydration	+	+	+	+	+	+	+	+	+	+
in situ PCR										
Polymerase	K	T	not		–	–	–	K	K	T
Primers *sul*1	+	+	perfor-		–	–	–	–	+	+
dUTP-Cy3	+	+	med		–	+	–	–	–	–
dNTPmix	+	+			–	–	–	+	+	+
Probe EUBmix	–	–			–	–	+	–	–	–
FISH										
Formamide, %	35	35	35	35	35	not		35	0–50	0–50
Probe EUBmix	+	+	+	+	+	per-		+	+	+
Probe SUL1	–	–	–	–	–	formed		+	+	+

K – Klenow-fragment polymerase; T – TAQ polymerase; * – agarose electrophoresis of PCR product were made

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After *in situ* PCR, the products were filtered (pore size 0.2 µm; Whatman) and then washed with 1 × PBS to remove remaining nucleotides and primers. FISH was performed on the filters as described in the previous section. In order to detect all bacteria a mixture of three probes: EUB338 I, EUB338 II and EUB338 III were used at the ratio 1:1:1 (Daims *et al.*, 1999). For series A, B, C and reaction d1 the formamide concentration in the hybridization buffer was 35%, while the reactions d2 and d3 were performed with a FA concentration of 0, 10, 20, 30, 40 and 50%. For microscopic observation each filter was placed on microscopic slide.

Quality of PCR products from reaction d2 and d3 was determined visually on a 1xTBE 0.8% agarose gel.

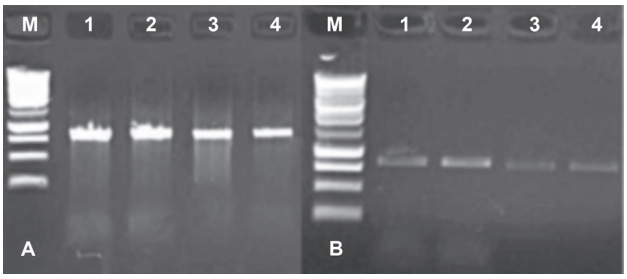

Fig. 1. Electrophoresis of PCR products

Figure A – summer samples, Figure B – winter samples; M – mass marker (100–1000 bp); position 1 and 3 – samples taken from nitrification tank, 2 and 4 – samples taken from settling tank; 1, 2 – primer set for *sul*1 (364 bp), 3 and 4 – primer set for *sul*2 (364 bp)

Results and Discussion

Presence of SMX resistance genes in activated sludge. Activated sludge taken for analysis in the study were sampled from two different seasons as we had doubts if the SMX resistance bacteria are present all over the year in a quantity suitable for detection of their genes. It is known that antimicrobial agents (as sulfonamides) present in the environment induce the resistance transfer among bacteria (Suzuki and Hoa, 2012) resulting in their better survival in harsh conditions. The concentration of sulfonamides in wastewater changes between the seasons resulting from higher use in seasons when the incidence of infection is greater and consumption of sulfonamides higher, thus in the winter (Bhandari *et al.*, 2008).

For detection of the genes in activated sludge bacteria, a DNA extraction and PCR were made using primers flanking the genes of interest. Agarose electrophoresis of the PCR products confirmed the presence of resistance genes *sul1*, and *sul*2 in samples taken from both the nitrification chamber and sedimentation tank, regardless of the season (Fig. 1). These genes enable bacteria to be insensitive to the antibiotic due to the production of the modified enzyme (encoded by these genes) dihydropteroate synthase (DHPS).

Previously reported data indicated presence of sulfonamide resistance genes among environmental bacteria in different aquatic compartments. Sulfamethoxazole resistance genes (*sul1*, *sul2* and *sul3*) were detected in shrimp ponds, a city canal and wastewater-feed fish ponds (Hoa *et al.*, 2008). *Sul1* and *sul2* were the most abundant SMX resistance genes. The predominance of *sul1* above *sul2* in wastewater and lake was mentioned earlier Czekalski *et al.* (2012). Genes *sulI* and *sul*II were identified in bacteria isolated from faeces in dairy farms (Srinivasan *et al.*, 2005), as well as in water and sediments from aquaculture (Pei *et al.*, 2006) and in the rivers (Mohapatrafv *et al.*, 2008) Gene *sulI* was also detected in sewage treatment plants, river water and seawater (Zhang *et al.*, 2009). Three genes carrying sulfamethoxazole resistance were also identified in sewage treatment plants in Germany (Szczepanowski *et al.*, 2009) and Poland (Łuczkiewicz *et al.*, 2013).

Detection of SMX resistance genes by FISH. Since no FISH probe had been designed and optimized, PCR primers were used as FISH probes to identify genes encoding sulfamethoxazole resistance (Table I). In order to estimate the conditions most suitable for hybridization, several hybridization conditions were tested, each time with different formamide concentration. Despite the presence of sulfamethoxazole resistance genes in activated sludge, no fluorescence signal originating from Cy3 was observed in all samples. Thus, we concluded that the detection of genes by FISH technique with the use of probes SUL1 and SUL2 had failed. The major limiting factor of the experiment was probably connected with small copy number of sulfamethoxazole resistance genes, resulting in signal intensity under the microscope detection level. While targeting 16S rRNA by FISH the detection limit is 10^3–10^4 cells. As the amount of resistance genes is much lower than that of rRNA the limit of their detection would be much higher. Techniques that intensify fluorescence signal, or increase the number of gene copies should be used to improve the results.

Detection of SMX resistance genes by *in situ* PCR and FISH technique. In order to amplify DNA, the fragment encoding sulfamethoxazole resistance, PCR was used together with both the non-labeled dNTPs and deoxyuridine triphosphate (dUTP) labeled with Cy3. dUTP-Cy3 was used for further visualization of the amplicons. To perform the PCR reaction the Klenow fragment of DNA polymerase I that is recommended for the synthesis of DNA with fluorescent nucleotide analogs was used. Besides Klenow fragment DNA polymerase, Taq polymerase was tested as well.

Since the detection of sulfamethoxazole resistance genes by traditional FISH failed, *in situ* PCR coupled with FISH was tested to reach the aim of this study. It

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Table III
Detection of fluorescence signal after *in situ* PCR-FISH

Series name*	A		B			C			D	
Reaction name*	a1	a2	b1	b2	b3	c1	c2	d1	d2	d3
Cy3 (red)	+	+				+		–	–	–
6-FAM (green)	–	–	+	–	+/–		–	–	–	–
Cy3/FLUOS	–	–						–	–	–

* see Table II for explanation of characteristics of series and reactions

was assumed that during the *in situ* PCR sulfamethoxazole resistance genes are amplified to an efficient level for microscopic observation. Subsequent FISH was performed on the post *in situ* PCR mixture. In this case universal bacterial probes were used to detect the sulfamethoxazole resistance genes inside the activated sludge bacteria.

Several attempts were made to optimize the *in situ* PCR-FISH conditions. The series characteristics are given in Table II, while the results are presented in Table III. The attempts were performed on all four samples treated as a repetitions. All results were the same for all the samples. Red signals were observed as a result of *in situ* PCR-FISH, suggesting a positive response from fluorochrome Cy3 used during *in situ* PCR (dUTP-Cy3) or from probe SUL1 used in FISH, while the green signal originated from 6-FAM that was used to label probe EUBmix. Observation of both green and red fluorescence signal (Cy3/FLUOS) at a particular position of a microscopic field (an overlay of fluorescence signals) was regarded as an amplicon found inside a bacterial cell.

After the first series (reaction a1 and a2), it was not clear if the Klenow fragment of DNA polymerase, or Taq polymerase is suitable for further study. During the microscopic analysis it was found that hybridization of post *in situ* PCR mixture with EUBmix had failed. There were only signals characteristic for fluorochrome Cy3, that labeled dUTP used to visualize amplicons formed during *in situ* PCR. The absence of green signal does not allow to confirm that the observed red signal comes from the amplified fragment *sul1*.

The series B aimed at finding the reasons for the lack of hybridization. Reactions were performed omitting *in situ* PCR step (reaction b1 and b2). As a result, it was found that the hybridization of activated sludge bacteria with the probe EUBmix occurred only when the samples were treated with lysozyme (reaction b1), which led to permeabilization of the cell wall so that it became permeable to the probe. There was no hybridization of activated sludge bacteria with the probe EUBmix if lysozyme and RNase were used together (reaction b2). The second series confirmed the suggestion that the use of RNase destroys the goal of hybridization – the genetic material (rRNA), and prevents proper identification of genes and microorganisms of interest. These series confirmed the suspicion that the use of RNase destroys the genetic material (rRNA), a goal during hybridization, and prevents the carrying out of this stage, and thus the identification of the microorganisms of interest. Thus, the protocol presented by Cheng and Hodson (2001) that was used in the study should be verified to be used for *in situ* PCR-FISH detection of genes in bacteria.

Subsequently, high temperature, which influences the activated sludge during *in situ* PCR, has an impact on the genetic material of activated sludge as well, and makes the hybridization difficult or impossible to carry out. The intensities of fluorescent signals observed after the hybridization were weak and close to the intensity of autofluorescence (background), while the activated sludge subjected to hybridization with probe EUBmix (allowing detection of all bacteria) without heating step resulted in a high intensity fluorescent signals derived from bacteria. Possibly the extra time or a change of formamide concentration in hybridization buffer would result in probe hybridization with genetic material of the activated sludge.

A third series (C), was designed to assess the possibility of the release of fluorochrome molecules during PCR (Table II). The reaction c1 covered the performance of thermal profile as used in PCR ("PCR" performed without a use of reaction mixture, polymerase and primers). It revealed the presence of strong red signals. The signal was probably a result of high temperatures used during PCR that caused a formation of fragile bonds between fluorochrome and dUTP. As a consequence, the release of the fluorochrome from dUTP and its binding to many cells or intracellular components had occurred (Cheng and Hodson, 2001). The red fluorescence signal that was observed originated from Cy3, and was not specific for the sulfamethoxazole resistance genes, although the genes could be amplified during *in situ* PCR. In comparison to reaction c1, in the reaction c2 probe EUBmix instead of dUTP was subjected to temperature profile to check the possibility of de-labeling of FLUOS from EUB probe. Microscopic observations of product of reaction c2 showed an absence of signals from green fluorochrome which labeled the EUBmix probe used in the reaction. The mechanism of binding between nucleotide and Cy3 is other than that for dUTP-Cy3, and the bond was more stable at higher temperature.

The fourth series (D), encompassed PCR reactions without the use of dUTP and followed by FISH. The FISH was performed with two probes – SUL1 and EUBmix to detect sequence of reverse primer used in *in situ* PCR and all bacteria, respectively. As a result no signals from the fluorochrome FLUOS (derived from EUBmix) and Cy3 (derived from SUL1) were observed

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during microscopic analysis. This indicated the absence of hybridization. In this series, the PCR products were verified by agarose gel electrophoresis. The product was found in the case of reaction d2 and d3. In the reactions, different polymerases were used. Reaction d1 (lack of dUTP and primers in the reaction mixture) served as the negative control. The presence of this product indicates that during the PCR reaction gene sul1 was amplified. This reaction was performed several times, each time with another formamide concentration in hybridization buffer (0–50%), to apply optimal hybridization conditions. Unfortunately, none of the tested reactions gave a positive result.

Summarizing the above, we failed to identify sulfamethoxazole resistance genes in nitrifying bacteria by the coupled *in situ* PCR – *in situ* hybridization techniques. However, this does not mean that this technique is useless in the identification of resistance genes but additional optimization is needed. We propose to treat the sludge only with lysozyme during the pretreatment step, extend the hybridization time, apply different concentrations of formamide in the hybridization buffer.

Other methods that could be used for the identification and visualization of resistance genes could be used as well. In order to perform detection based on FISH and primers flanking the investigated resistance gene as oligonucleotide probes the fluorescent signal must be significantly strengthened to detect a small number of copies. For this purpose the DOPE-FISH or CARD-FISH were designed. DOPE-FISH (Double Labeling of Oligonucleotide Probes for Fluorescence *In Situ* hybridization) is a FISH performed with the use of double labeled probes – at the 5 'and 3' end of tested fragment (Stoecker *et al.*, 2010). CARD-FISH (catalyzed reporter deposition-fluorescence *in situ* hybridization) represents a method in which signal amplification is caused by use of an enzyme (Bobrow *et al.*, 1989). The FISH probe is labeled with horseradish peroxidase (HRP). After the hybridization the sample is incubated with fluorescently labeled thyramid. The activity of HRP results in production of radical form of thyramid and its accumulation in a cell, close to hybrid with probe thus amplifying the fluorescence signal. However, application of this method requires careful and controlled permeabilization step to avoid disturbance of the cell structure by diffusion of large size probe. Intensification of the fluorescent signal during the application of *in situ* hybridization can be achieved using the so-called RING-FISH (Recognition of Individual Genes in a Single Bacterial Cell by Fluorescence *In Situ* hybridization) – the variety of traditional FISH method used to identify individual genes. The modification involves the use of polynucleotide probes labeled every 10–20 nucleotides and their use in high concentrations (approximately 250 ng/ml), addition of denaturation step before hybridization and extension of hybridization time beyond 24 h. The fluorescence signal obtained is visible as a distinctive ring. It is recommended to apply several times the labeled probe designed for DNA microarray as probes for the identification of resistance genes (Majtan *et al.*, 2007). RING-FISH was successfully applied to the detection of beta-lactam resistance genes on plasmids (Zwirglmaier *et al.*, 2004). This method is not often used, probably because of the considerable cost of polynucleotide probes, which lies in the difficulty of chemical labeling of the probe at least every 10 nucleotides. The authors of the publication ordered such a polynucleotide probe in two biotech companies. We wanted two polynucleotide fragments (with a length of 50 and 60 nucleotides) to be synthesized. There were oligonucleotides sul1 and sul2 used for microarray (van Hoek and Aarts, 2008). After several attempts of synthesis the companies cancelled the order completion excusing themselves by the impossibility of labeling the fragment 5–6 times. They were able to provide fragments of interest labeled maximum 3-times. A kind of compromise could be the generation of own polynucleotide probes labeled fluorescently through molecular genetics. Biotech companies recommend amplification and labeling of DNA during PCR. The purchase of a matrix in the form of a polynucleotide fragment of the desired DNA sequence, followed by its amplification in the presence of labeled nucleotides is then necessary. This method does not give any assurance that the labeled nucleotides are further apart than every 10 nucleotides, which is relatively important because the shorter distance between the fluorochromes causes quenching of fluorescent signal.

Conclusions. Sulfamethoxazole resistance genes were detected in activated sludge bacteria by PCR. Their visualization by FISH failed probably due to a low number of gene copies. To enrich their number and visualize them by microscopic techniques inside the bacterial cells a hybrid of *in situ* PCR and FISH was used. Performance of *in situ* PCR under activated sludge bacteria with the use of fluorescently labeled nucleotides was designed to amplify the resistance genes and simultaneously label them with a fluorescent dye.

In situ PCR and subsequent FISH were used to multiply certain resistance genes and detect and visualize them in bacterial cells by microscopic observations. The idea that FISH may be performed on post-*in situ* PCR mixture has been verified by detection of resistance genes in activated sludge bacteria. Detection of sulfamethoxazole resistance genes via *in situ* PCR and fluorescence *in situ* hybridization was not possible. After several attempts first we could not accomplishj labeling of amplicons during PCR and secondly hybridization following PCR.

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