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Detection of antibiotic resistance genes in wastewater treatment plant – molecular and classical approach

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Abstract: Antibiotics are a group of substances potentially harmful to the environment. They can play a role in bacterial resistance transfer among pathogenic and non-pathogenic bacteria. In this experiment three representatives of medically important chemotherapeutics, confirmed to be present in high concentrations in wastewater treatment plants with HPLC analysis were used: erythromycin, sulfamethoxazole and trimethoprim. Erythromycin concentration in activated sludge was not higher than 20 ng L^{-1}. N-acetylo-sulfamethoxazole concentration was 3349 ± 719 in winter and 2933 ± 429 ng L^{-1} in summer. Trimethoprim was present in wastewater at concentrations 400 ± 22 and 364 ± 60 ng L^{-1}, respectively in winter and summer. Due to a wide variety of PCR-detectable resistance mechanisms towards these substances, the most common found in literature was chosen. For erythromycin: *erm* and *mef* genes, for sulfamethoxazole: *sul1*, *sul2*, *sul3* genes, in the case of trimethoprim resistance *dhfrA1* and *dhfr14* were used in this study. The presence of resistance genes were analyzed in pure strains isolated from activated sludge and in the activated sludge sample itself. The research revealed that the value of minimal inhibitory concentration (MIC) did not correspond with the expected presence of more than one resistance mechanisms. Most of the isolates possessed only one of the genes responsible for a particular chemotherapeutic resistance. It was confirmed that it is possible to monitor the presence of resistance genes directly in activated sludge using PCR. Due to the limited isolates number used in the experiment these results should be regarded as preliminary.

Introduction

Pharmaceuticals, including antibiotics, are an important and diverse group of substances potentially harmful to the environment. They are necessary in bacterial disease treatment but their inappropriate or overusage can cause bacterial resistance (Wise 2002). Interestingly, not the hospitals but single households are the main source of antibiotics in the environment. It was estimated that antibiotics worldwide consumption is ca. 0.1–0.2 Mt yr^{-1} (Wise, 2002).To this, there is use of them in aquaculture, bee-keeping and fruit-farming (Kümmerer 2009). In their "lifecycle" antibiotics and/or their metabolites are directed to the wastewater treatment plants and then to water and further to the other parts of the environment.

Antibiotics resistance is a timely topic due to the possibility of its transfer among pathogenic and non-pathogenic bacteria (Rahube and Yost 2010). This phenomenon is possible because the resistance genes are often located on mobile genetic elements, such as plasmids, which appear commonly in bacteria-rich areas such as wastewater treatment plants (WWTPs). From WWTPs they are directed to the water tracts becoming a danger for public health. The monitoring of antibiotic resistance presence and transfer in activated sludge seems to be reasonable part of the antibiotics usage politics. These analyses can be performed with traditional cultivation methods but more often than not, the molecular approach based on PCR (polymerase chain reaction) is used due to the higher sensitivity and precision of the test. More than 95% of the environmental bacteria cannot be cultivated in the laboratory (Oliver 2010). That is the other reason why traditional microbiological methods should be supported with molecular tools.

In this experiment three representatives of medically important chemotherapeutics and antibiotics confirmed to be present in high concentrations in WWTPs: erythromycin, sulfamethoxazole and trimethoprim were chosen. These substances are very popular in Poland for bacterial diseases treatment and their concentration in wastewater is high especially during autumn-winter season.

Erythromycin is a macrolide antibiotic that has an antimicrobial spectrum slightly wider than that of penicillin and used more often in bacterial infection treatment. Microorganisms possess several resistance mechanisms towards erythromycin,

such as a modification of the antibiotic-ribosome binding place by methylation/mutation or active erythromycin removal from bacterial cell by transporting proteins located in cell membrane or enzymatic inactivation (Weisblum 1995). The genetic control of the erythromycin resistance is based both on chromosomal and plasmid-coded information. Methylases are encoded by plasmid-located *erm* genes. These genes determine multiresistance towards macrolides, lincosamides and streptogramins B. They are spread among Gram-positive and Gram-negative bacteria. Chromosomal or plasmid-located *mef* and *msr* genes encode active erythromycin efflux (Matsuoka et al. 2003). Mef proteins appear mainly among Gram-positive bacteria. These genes are easily transferable among genus and species. The most common resistance mechanisms are binding site modification and active antibiotic removal, that is why in this experiment *erm* and *mef* genes were chosen to monitor erythromycin resistance in activated sludge.

Sulfamethoxazole is a sulfonamide antibiotic in which bactericidal efficacy increases in combination with trimethoprim – a bacteriostatic chemotherapeutic agents known as dihydrofolate reductase inhibitor (DHPS) (Huovinen 2001). Because these two drugs are used as a combination with synergic effect their resistance mechanisms when separately analyzed are difficult to distinguish. The most reasonable theory as to how bacteria gain resistance towards the combination of these two drugs is probably through the permeability barrier and/or efflux pumps (Huovinen 2001). Sulphonamides resistance is probably encoded with plasmid-located *sul* genes. Two types of this gene – *sul1* and *sul2*, are commonly present in Gram-negative bacteria and they are detected in resistant microorganisms in comparable frequency (Sköld 2001, Perreten and Boerlin 2003). *Sul1* and *sul2* are similar to each other in 57%, but their origin is still unknown. In 2003 *sul3* gene presence in *E. coli* strain isolated from pigs in Switzerland was reported (Perreten and Boerlin 2003).

Trimethoprim resistance is mainly based on modified dihydrofolate reductase production encoded by *dhfr* genes. We actually know 30 types of *dhfr* gene, linked with integrons, transposons and plasmids (Brolund et al. 2010). The most common in Gram-negative bacteria is *dhfrA1*, appearing in the integron cassettes class 1 and 2, which could be located on plasmid or chromosome (Sköld 2001).

The aim of this study was to assess the possibility of fast PCR detection of resistance genes towards erythromycin, sulfamethoxazole and trimethoprim in both activated sludge and pure strains isolated from a communal wastewater treatment plant in Zabrze (Poland). Resistant bacteria were also enumerated with the microbiological plating method. Activated sludge samples collected in four seasons within one year underwent PCR-DGGE (polymerase chain reaction--denaturing gel electrophoresis) analysis to estimate the genetic changeability of the biocenosis. HPLC (high pressure liquid chromatography) was used to detect the antibiotic concentrations chosen for the experiment.

Materials and methods

HPLC – based determination of the antimicrobial agents presence in the wastewater treatment plant

The presence of antimicrobial agents in the wastewater treatment plant was determined by means of HPLC method combined with MS detection in the influent and the effluent of the WWTP. The samples (volume of 1 L) were collected three times per season from bioreactor and from return sludge containers. These locations were chosen as a potential reservoir of antimicrobial agents and resistance genes. The samples were frozen and stored in dark-glass bottles at – 20ºC until analysis. After defrosting, samples were filtered through glass fiber filters (pore size of < 1 µm, diameter 55 mm; Dassel, Germany). One hundred mL of the influent and 200 mL of the effluent were spiked with a mixture of internal standards (IS). The pH of all samples was adjusted to 7.5. As the internal standards the following substances were used: sulfamerazine-d4 (IS for sulfonamides and trimethoprim), sulfamethoxazole-d4 (IS for sulfamethoxazole; Sigma--Aldrich), N-acetyl-sulfamethoxazole-d5 (IS for N-acetyl--sulfamethoxazole; Sigma-Aldrich) and (E)-9-[O-(2-methyloxime)]-erythromycin (IS for macrolides; synthesized according to the procedure described previously (Schluesener et al. 2003). The solid phase extraction of the wastewater samples was performed using Oasis HLB (200 mg, 6 mL) cartridges (Waters, Milford, MA, USA). Conditioning and drying of the cartridges were performed as previously described (Hjosa-Valsero et al. 2011). The analytes were eluted with 4 × 2 mL acetone. The eluates were concentrated to the volume of 200 µL, diluted with 250 µL of methanol and evaporated under a gentle nitrogen stream to the volume of 100 µL. Then the extracts were redissolved in 400 µL Milli-Q water. All the samples were analyzed by reversed--phase liquid chromatography-tandem mass spectrometry (HPLC-MS-MS). The HPLC system consisted of a G1313A autosampler, a G1311A quaternary HPLC pump, a G1379A degasser (all Agilent, Waldbronn, Germany), a CTO-10A column oven and a SCL-10A system controller (all Shimadzu, Duisburg, Germany). More details concerning chromatographic analysis performance are presented in (Hjosa-Valsero et al. 2011).

Enumeration and isolation of antibiotic resistant bacteria in activated sludge

Activated sludge samples were collected from municipal WWTP in Zabrze (Poland) three times per sampling time from bioreactor and return sludge container. The samples were diluted in sterile 0.9% NaCl to 10^{-12} and the suspension was used to inoculate agar plates containing Mueller--Hinton medium (BTL, Poland) with sulfamethoxazole, trimethoprim and erythromycin in concentration of 5 µg mL^{-1} each (Polfarma, Poland). Mueller-Hinton plates without antibiotics were used as a control. The plates in triplicate from each dilution were incubated for 24–48 h in 20±1°C. Bacteria growing on particular antibiotic plates and on the control plates were counted and several morphological bacteria types were selected for pure strain isolation. The isolated strains underwent morphological identification with Gram staining.

Minimal Inhibitory Concentration (MIC) value estimations for isolated bacteria

The estimations of MIC of antibiotics used in the experiment: erythromycin, sulfamethoxazole and trimethoprim were performed in liquid broth medium (BTL, Poland) with antibiotics in concentration of: 4–1024 µg mL^{-1} (Martin et

al. 2001). The 24 h colonies of the isolates were analyzed for MIC value in triplicate. The samples were incubated for 24 h in 26°C.

DNA extraction and PCR conditions for activated sludge samples prepared for DGGE

Activated sludge samples (volume of 10 mL) Zabrze WWTP were pelleted by centrifugation (10 000 rpm, 10 min, 4°C) and stored at -20°C. Total genomic DNA was extracted from 0.2 g of the activated sludge samples with mechanical method. The samples washed with 1 × PBS buffer (Sigma) were disintegrated with bead beating (Roth, Germany) in lysis buffer containing Tris-HCl 100 mM, EDTA 100 mM and NaCl 1.5 M (pH = 8.0). The samples were incubated 20 min in 1400 rpm and 200 µL 10% SDS was added. After 30 min of incubation in 65°C samples were centrifuged twice at 13 000 rpm and placed on spin filters (A&A Biotechnology). DNA attached to the filter was washed twice with 70% ethanol solution (A&A Biotechnology). The amount of DNA was measured spectrophotometrically using Qubit (Invitrogen) and stored at -20°C until PCR amplification.

In this study partial 16S rRNA gene amplification was performed using primers 338F with GC clamp and 518R, which amplified a partial (ca. 180 bp) 16S rRNA gene fragment of all the bacteria (Muyzer et al. 1993). PCR procedure was described previously (Ziembińska et al. 2009).

Denaturing gradient gel electrophoresis conditions and results analysis

The DGGE of the PCR products obtained in reactions with 338F-GC and 518R primers underwent electrophoretic separation in the Dcode Universal Mutation Detection System (BioRad). Polyacrylamide gel (8% for 16S rRNA gene, 37:1 acrylamide-bisacrylamide, Fluka) with a gradient of 30–60% denaturant was prepared according to the manufacturer's instruction. The gel was run for 10 h at 70 V in a 1 × TAE buffer (Tris, acetic acid, EDTA, pH = 8.0) at a constant temperature of 60°C. The gel was stained with SYBR Gold (1:10 000, Invitrogen) in MiliQ water for 30 min and distained in MiliQ water for 40 min, then visualized under UV light and photographed using Quantity One 1D (BioRad).

The analysis of DGGE fingerprints was performed using a Quantity One 1D software (BioRad). Bacterial biodiversity was estimated on the basis of densitometric measurements and Shannon diversity index was calculated (Ziembińska et al. 2009).

DNA extraction form pure strains of antibiotic resistant bacteria isolated from activated sludge and PCR conditions for resistance genes identification

DNA from resistant isolates to erythromycin and sulfametoxazole/trimethoprim were extracted using DNA Genomic Mini Kit (A&A Biotechnology). The amount of DNA was measured spectrophotometrically using Qubit (Invitrogen) and stored at -20°C until PCR amplification. PCR mixture volume of 30 µL was containing: 1× polymerase buffer (Promega), 2 mM $MgCl_2$ (Promega), 5 pmol dNTPs (Promega), 20 pmol of each primer (Genomed), 1.5 U GoTAQ Flexi Polymerase (Promega) and 0.15–0.2 µg μL^{-1} DNA. The DNA samples from resistant bacterial pure strains and total bacterial DNA from activated sludge were used as the templates.

The primers used in the study were targeting *erm* and *mef* genes responsible for erythromycin resistance, *sul1*, *sul 2* and *sul 3* genes encoding sulfametoxazole resistance, and *dhfrA1* and *dhfrA14* genes used to detect trimethoprim resistance. Primers sequences and PCR amplification conditions are enlisted in Table 1 and 2, respectively. The products after PCR amplification were separated in 0.8% (w/v) agarose gel (Promega) in 1×TBE buffer (Tris, boric acid, EDTA, pH = 8.3) with ethidium bromide (10 mg mL^{-1}; Promega), visualized under UV light and photographed.

Results

HPLC analysis of antibiotics concentration

HPLC analysis of raw and treated wastewater from Zabrze WWTP enabled the estimation of the concentration and

Table 1. Primers used in the study

Primer	Primer's sequence 5'–3'	References
ermF	5' GAAAGGTACTCAACCAAATA 3'	Sutcliffe et al. 1996
ermR	5' AGTAACGGTACTTAAATTGTTTAC 3'	
mefF	5' AGTATCATTAATCACTAGTGC 3'	
mefR	5' TTCTTCTGGTACTAAAAGTGG 3'	
sul1F	5' ATGGTGGACGGTGTTCGGCATTCTGA 3'	Toleman et al. 2007
sul1R	5' CTAGGCATGATCTAAACCCTCG 3'	
sul2F	5' GAATAAATCGCTCATCATTTTCGG 3'	
sul2R	5' CGAATTCTTGCGGTTTCAGC 3'	
sul3F	5' GAGCAAGATTTTTGGAATCG 3'	Grape et al. 2003
sul3R	5' CATCTGCAGCTAACCTAGGGCTTTGGA 3'	
dhfrA1F	5' CCAAAGGTGAACAGCTCCTG 3'	Hoek et al. 2005
dhfrA1R	5' ATATGTAGTGTATCTACTTG 3'	
dhfrA14F	5' TCTGGTGGGTCGCAAAGACG 3'	
dhfrA14R	5' ATGGGTAATGTTTCTCGG 3'	

Table 2. PCR amplification programs used in the study

Primers set / PCR step	*erm*	*mef*	*dhfrA1*	*dhfrA14*	*sul1*	*sul2*	*sul3*
Pre-denaturation	93°C/3 min	93°C/3 min	95°C/3 min	95°C/3 min	95°C/5 min	95°C/5 min	95°C/5 min
Denaturation	93°C/ 1 min	93°C/ 1 min	95°C/ 30 sec	95°C/ 30 sec	95°C/ 15 sec	95°C/ 15 sec	95°C/ 15 sec
Annealing	52°C/ 1 min	52°C/ 1 min	55°C/ 30 sec	55°C/ 30 sec	51°C/ 30 sec	51°C/ 30 sec	51°C/ 30 sec
Elongation	72°C/ 1 min	72°C/ 1 min	72°C/ 30 sec	72°C/ 30 sec	72°C/ 1 min	72°C/ 1 min	72°C/ 1 min
	×35	93°C/ 1 min, 52°C/ 1 min, 72°C/ 1 min ×35	×35	×35	×30	×30	×30
Final elongation	72°C/5 min	72°C/5 min	72°C/10 min	72°C/10 min	72°C/7 min	72°C/7 min	72°C/7 min
References	Sutcliffe et al. 1996		Hoek et al. 2005		Hoa et al. 2008, Hoek et al. 2005		

the removal percentage of the antibiotics used in the experiment. These values were calculated for erythromycin, sulfamethoxazole, N-acetylosulfamethoxazole (the main sulfamethoxazole metabolite) and trimethoprim for winter (T=10ºC) and summer (T=20ºC) sampling period (temperature measured in the nitrification chamber of WWTP). Erythromycin is able to absorb onto the surface of raw wastewater sludge flocs and during the biological treatment of wastewater it can be desorbed into the liquid phase. All analyses were conducted in filtered wastewater, therefore erythromycin concentration in the raw wastewater as the removal level could not be calculated. The highest removal was obtained for N-acetylosulfamethoxazole (94 and 99% for winter and summer, respectively), while trimethoprim was removed at the lowest level (8 and 22% for winter and summer, respectively). The results of the HPLC analysis are shown in Table 3.

Enumeration of antibiotic resistant bacteria in activated sludge

Traditional microbiological plating used for bacterial enumeration is not precise but it is useful for rough calculation of bacterial number changes during the experiment. The total number of bacteria during the year was changing from 12×10^9 mL^{-1} in summer to 42×10^9 mL^{-1} in winter (Figure 1a). The increase of bacterial number was observed in winter-spring season. Similar tendency was observed in case of erythromycin resistant bacteria, with the highest number in winter. The number of bacteria resistant towards sulfamethoxazole/trimethoprim was also the lowest in the summer, but the range of changes in case of sulfamethoxazole/trimethoprim resistant bacteria was less drastic during autumn – spring season (Figure 1b).

Gram staining and MIC values of isolated antibiotic resistant strains

The bacterial isolates resistant towards the antibiotics used in the experiment were obtained from activated sludge in Zabrze WWTP underwent Gram staining. In case of erythromycin resistant bacteria (Table 4) most of the isolates were Gram-negative. Only three strains (E1, E5 and E8) were identified as Gram-positive. Seven isolates presented very high MIC values (>1024 μg mL^{-1}), while MIC values only for two strains were lower: for E3 was 256 μg mL^{-1} and for E7 was 512 μg mL^{-1}.

Sulfamethoxazole/trimethoprim resistant bacteria isolated from activated sludge in Zabrze WWTP presented very high MIC values (all strains MIC values were above >1024 μg mL^{-1}). Eight out of nine isolated were Gram-negative (Table 4).

PCR-based detection of antibiotic resistance genes and total biodiversity of the community analysis

The antibiotic resistance genes (*emr, mef, sul1, sul2, sul3, dhfrA1, dhfrA14*) can be detected in activated sludge as well as in pure strains using PCR amplification. Such a procedure enabled the resistance genes to be identified both in isolated strains known to be resistant to a particular antibiotic and in the activated sludge samples from which the pure strains were obtained. The PCR amplicons were separated electrophoretically and visualized under UV. Optimized PCR was performed twice, with comparable result.

Figure 2a presents PCR products obtained for *erm* gene and Figure 2b for *mef* gene amplified in the erythromycin resistant strains E1–E9 and activated sludge sample. Table 4 (part A) presents the comparison of *erm* and *mef* genes presence in the erythromycin resistant strains E1–E9 and activated sludge sample.

The Figures 3a and 3b respectively present the results of *dhfrA1* and *dhfrA14* genes amplification in bacterial resistant strains B1–B9 and in the activated sludge sample. The results for *sul1* and*sul2* genes PCR amplification for bacterial resistant strains B1–B9 and in activated sludge sample are shown in the Figures 4c and 4d, respectively. No *sul3* gene was detected in this study. The comparison of *sul* and *dhfr* genes presence in sulfamethoxazole/trimethoprim resistant bacterial strains and in the activated sludge from which these strains were isolated is presented in Table 4 (part B).

The PCR products of a 16S rRNA gene fragment (length ca. 180 bp) obtained using 338F-GC and 518R primers were separated in polyacrylamide gel with 30–60% denaturant gradient. The result was visualized in UV light (Figure 4a) and on the basis of densitometric analysis Shannon diversity index was calculated (Figure 4b).

Table 3. The concentration and the removal percentage of selected antimicrobial agents in winter (T = 10°C) and summer (T = 20°C) at WWTP in Zabrze

Antimicrobial agent	Temperature	Concentration in the raw wastewater [ng L^{-1}]	Concentration in the treated wastewater [ng L^{-1}]	Removal [%]
sulfamethoxazole	*T=10°C*	1745 ± 253	1003 ± 146	43
	T=20°C	1778 ± 274	1226 ± 205	31
N-acetylo-sulfamethoxazole	*T=10°C*	3349 ± 719	196 ± 47	94
	T=20°C	2933 ± 429	39 ± 18	99
trimethoprim	*T=10°C*	400 ± 22	369 ± 29	8
	T=20°C	364 ± 60	283 ± 49	22
erythromycin	*T=10°C*	n.d.	16 ± 2	n.r.
	T=20°C	n.d.	17 ± 2	n.r.

n.d. – not detected
n.r. – no removal

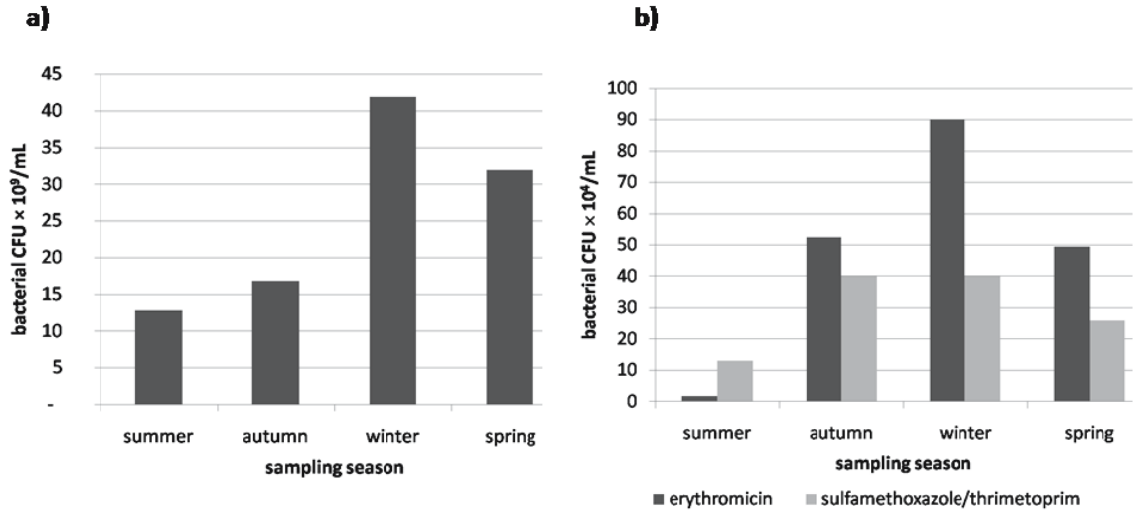

Fig. 1. Bacteria enumeration in activated sludge, performed with a plating method on Mueller-Hinton medium for four sampling seasons; a) total bacterial number; b) the number of erythromycin and sulfamethoxazole/trimethoprim resistant bacteria

Table 4. Gram staining and MIC value results erythromycin and sulfamethoxazole/trimethoprim resistant bacteria isolated from Zabrze WWTP activated sludge with the comparison of *erm* and *mef* genes presence in the erythromycin resistant strains E1–E9 and activated sludge (AS) sample (part A) and *sul* and *dhfr* genes presence in the sulfamethoxazole/trimethoprim resistant strains B1–B9 and activated sludge (AS) sample (part B)

PART A										
Strain	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**	**E9**	**AS**
***Erm* gene presence**	+	+	–	+	+	+	–	+	+	+
***Mef* gene presence**	+	–	–	+	–	–	–	–	–	+
Gram staining	+	–	–	–	+					x
MIC [µg mL^{-1}]	>1024	>1024	256	>1024	>1024	>1024	512	>1024	>1024	x
PART B										
Strain	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**AS**
***dhfrA1* resistance gene**	–	–	+	–	+	–	–	–	+	+
***dhfrA14* resistance gene**	–	–	–	–	+	–	–	+	+	+
***sul1* resistance gene**	+	+	+	+	+	+	–	–	+	+
***sul2* resistance gene**	–	–	+	+	+	–	–	–	+	+
***sul3* resistance gene**	–	–	–	–	–	–	–	–	–	–
Gram staining	–	–	–	–	–	–	+	–	–	x
MIC [µg mL^{-1}]	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	x

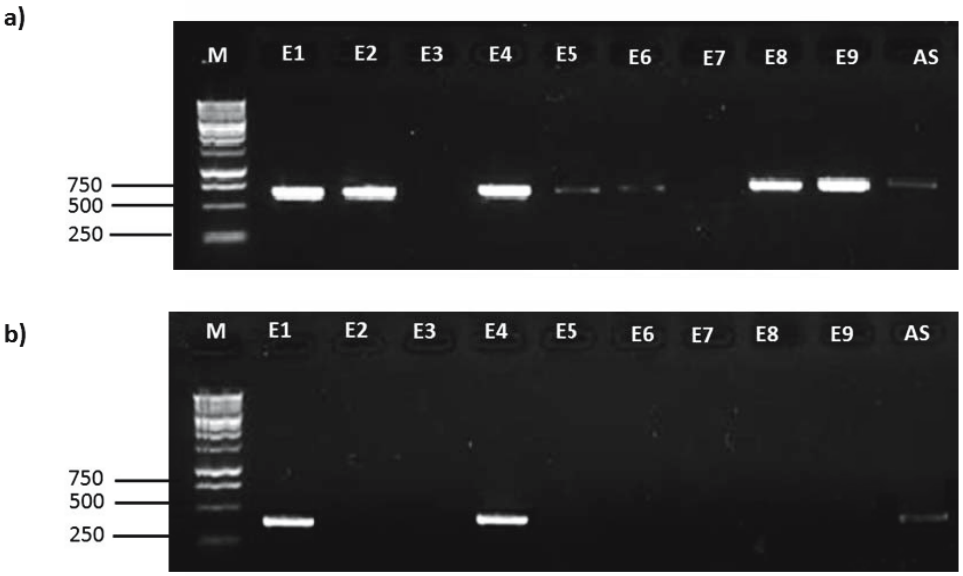

Fig. 2. PCR products obtained for (a) *erm* gene for the erythromycin resistant strains E1–E9 and activated sludge sample (AS); (b) *mef* gene for the erythromycin resistant strains E1–E9 and activated sludge sample(AS); M – 1 kb ladder (Promega)

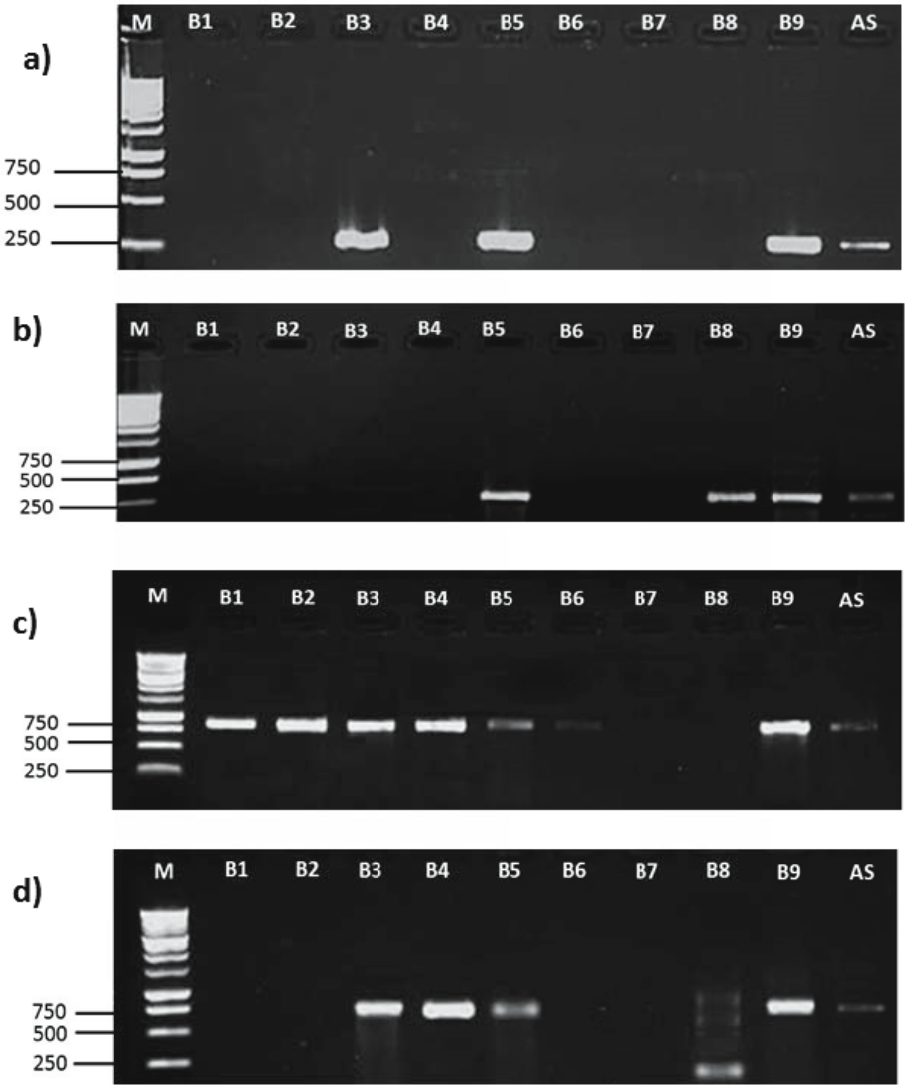

Fig. 3. PCR products obtained for: (a) *dhfrA1* genes for the trimethoprim resistant strains B1–B9 and activated sludge sample (AS); (b) *dhfrA14* genes for trimethoprim resistant strains B1–B9 and activated sludge sample (AS); (c) *sul1* genes for sulfamethoxazole resistant strains B1–B9 and activated sludge sample(AS); (d) *sul2* genes for sulfamethoxazole resistant strains B1–B9 and activa ted sludge sample(AS); M-1 kb ladder (Promega)

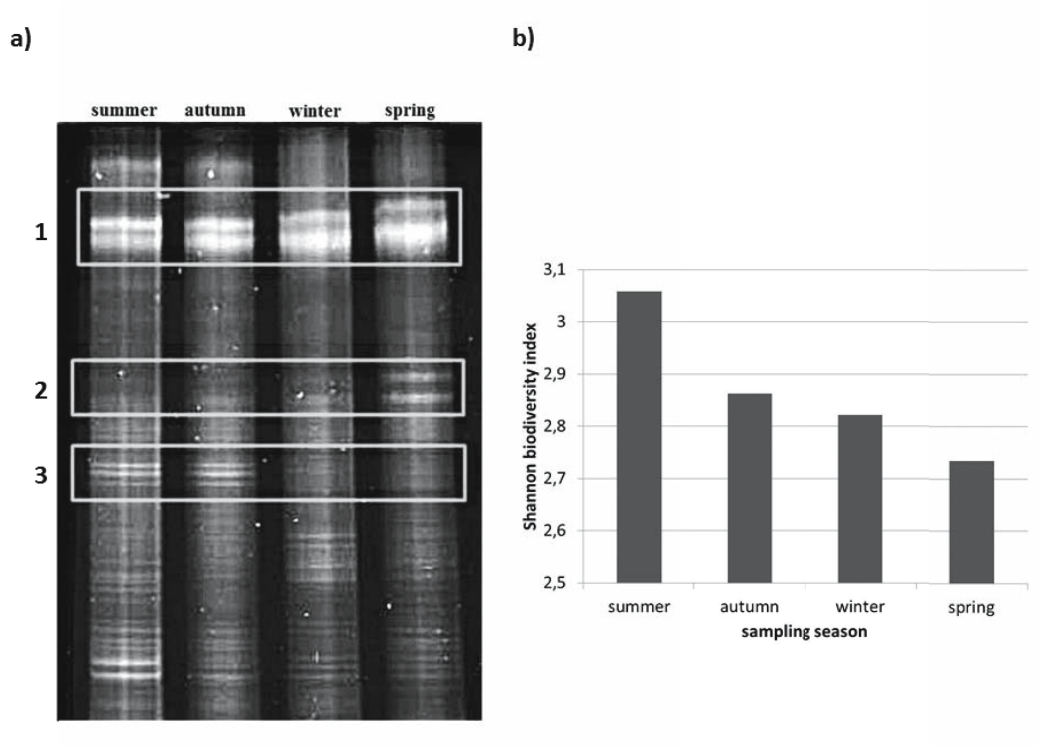

Fig. 4. (a) DGGE fingerprints obtained for activated sludge samples collected in summer, autumn, winter and spring season; PCR fragment length ca. 180 bp was separated in polyacrylamide gel with 30–60% denaturant (frame 1 – genotypes present during total length of the experiment; frame 2 – genotyped appearing gradually; frame 3 – genotypes disappearing gradually); (b) Shannon diversity index changeability calculated on the basis of densitometric analysis of DGGE picture for activated sludge samples collected in summer, autumn, winter and spring season

Discussion

The natural antibiotic substances have been used for ages. Bacteria, the antibiotic target, are able to gain resistance against particular antibiotic substances by the exchange of their genetic material in horizontal gene transfer (HGT). That is why the research to estimate the level, cause and range of antibiotic resistance in the environment is very important. Activated sludge in biological WWTPs is an excellent source for resistance genes, as well as a very good experimental biocenosis for resistance transfer analysis. In this experiment we try to correlate the levels of three antimicrobial compounds: erythromycin, sulfamethoxazole and trimethoprim, measured in communal WWTP in Zabrze, Poland with the occurrence of these antibiotics resistance genes in activated sludge analyzed with PCR. To evaluate this tool for the resistance monitoring in the environment we isolated erythromycin and sulfamethoxazole/trimethoprim resistant bacterial strains (nine each) from an activated sludge, to confirm the presence of these genes, both in pure strains and the activated sludge samples. In order to observe the changeability of antibiotic resistant bacteria level in activated sludge during the year, plating analysis was performed. The activated sludge samples collected in four seasons underwent PCR-DGGE analysis to present the genetic structure and changeability of activated sludge biocenosis.

According to ESAC (European Surveillance of Antimicrobial Consumption) in Poland in 2008 macrolides (together with lincosamides and streptogramidins it was 17.7% of total antibiotics consumption) were second the most popular group of antibiotics used in bacterial diseases treatment. Erythromycin was not detected in raw wastewater while in treated wastewater its concentration was not higher than 20 ng L^{-1} (Table 3). It could be explained that this antibiotic is present in the wastewater as its derivatives converted to erythromycin in WWTP conditions. Additionally, it can be adsorbed on suspended solids in the sewage and all the analyses were performed on filtrated wastewater, so the tests could give lowered levels of erythromycin concentration. No analyses of erythromycin derivatives were performed. It was also mentioned in the literature (Goebel et al. 2004) that erythromycin can easily convert into erythromycin – H_2O form during sample preparation. This form of the antibiotic was also not analyzed in the experiment.

Sulfamethoxazole is one of the most popular sulphonamides in Poland. Its concentrations were comparable in both summer and winter season. The results obtained in the experiment suggests that this substance in better removed in winter (43%), than in summer (31%) (Table 3). According to the bibliographic data (Goebel et al. 2004) sulfamethoxazole should be balanced together with its main derivative – N-acetylo-sulfamethoxazole, because in biological WWTPs the conversion back to sulfamethoxazole is often observed, regardless of the temperature. Thus the level of sulfamethoxazole removal together with its metabolite is at a comparable level in both experimental seasons. N-acetylo--sulfamethoxazole was the substance with the highest concentration in raw wastewater (3349 ± 719 in winter and 2933 ± 429 ng L^{-1} in summer). Trimethoprim used together with sulfamethoxazole was present in raw wastewater in lower

concentration (400 ± 22 and 364 ± 60 ng L^{-1}, respectively in winter and summer). Trimethoprim was practically non removable (8%) in winter, while in summer its removal was estimated to be ca. 22%.

The total number of bacteria during the year was changing with the increase of bacterial number in winter-spring season (Figure 1a). Probably such a situation was observed because the biomass in the bioreactor was higher in order to maintain the effectiveness of wastewater treatment process in the lower temperatures. The same tendency was observed in case of erythromycin resistant bacteria, with the highest number in winter. The increase of the number of resistant bacteria could be caused by the higher usage of this antibiotic to treat bacteria diseases (Figure 1b), because as it was previously stated, the exposition to the drug can cause the increase of bacterial resistance (Silva et al. 2006, Soufi et al. 2010). Because erythromycin is known to possess wider bactericidal spectrum and it is used more often than sulfamethoxazole/trimethoprim, it is possible that its usage increased during winter. During this season the morbidity is higher and it caused the increase of resistant bacteria number in activated sludge. These results cannot be confirmed with HPLC results of erythromycin concentration in raw wastewater directed to the activated sludge tank because as mentioned earlier, samples preparation causes problems with analysis.

The number of bacteria resistant towards sulfamethoxazole/trimethoprim was also the lowest in the summer (Figure 1b), but the changeability range of sulfamethoxazole/trimethoprim resistant bacteria number was less drastic during autumn-spring season. Such a situation can be explained by sparser usage of these substances in health care in Poland. These drugs are more often used for urinary tracts than for respiratory tracts problems treatment and these health problems are seasonally variable. It should be also mentioned that trimethoprim removal during winter is lower, so bacteria are exposed to its higher dosages in WWTP. Resistance genes acquisition can be caused by longer exposition.

These data can be partially confirmed by DGGE results. As it can be seen in Figure 4a, some of the genotypes appear in winter – spring time, while more disappear. The total biodiversity decreases in winter – spring period and it could be suspected that the resistant bacteria are the genotypes constantly present and appearing in the fingerprint pattern. Biodiversity was decreasing from summer to spring, which suggests that the temperature was the most important factor for bacterial number changeability with higher multiplication in the higher temperatures during summer (Figure 4b). Interestingly, the biodiversity was decreasing but in the same time the number of bacteria estimated with plating method was increasing (Figure 1a). It is possible that only a few genotypes of bacteria were capable of multiplying during winter-spring season. AT-rich genotypes are present in DGGE patterns regardless to the sampling season (Figure 4a, Frame 1), while GC-rich genotypes number is changing (Figure 4a, Frame 2 and 3). It could be possible that GC-rich genotypes belong to resistant bacteria but this hypothesis requires further research.

Most of the activated sludge bacteria are Gram-negative (Forster et al. 2002). As it was suspected erythromycin resistant strains isolated in the experiment were mainly Gram-negative (Table 4). Only three strains (E1, E5, E8) were Gram-positive. Gram-negative bacteria possess an additional outer membrane impermeable towards a large number of toxic compounds which make them resistant towards harmful substances present in the environment. MIC values of the isolated erythromycin resistant strains are high. For seven of nine isolates minimal inhibitory concentration of the antibiotic is higher than 1024 μg mL^{-1}. Only strains E3 and E7 presented lower resistance, their MIC values are 256 and 512 μg mL^{-1}, respectively. According to the previous research (Schönberg-Norio et al. 2006) MIC higher than 1024 μg mL^{-1} characterizes clinical isolates. Activated sludge bacteria, as the clinical isolates, are in constant contact with the antibiotics which can be the cause of resistance development (Szczepanowski et al. 2004).

Seven isolates – E1, E2, E4, E5, E6, E8 and E9 – possess *erm*B gene only (Figure 2, Table 4). In activated sludge both genes – *mef* and *erm* are present. According to the data obtained it could be stated that the main erythromycin resistance mechanism is enzymatic, posttranslational modification of 23S rRNA, being the target for this antibiotic as the effect of *ermB* encoded N-methylotransferase activity. These results confirm previous bibliographic data suggesting that this mechanism is the most frequent erythromycin-resistance mechanism (Artur et al. 1990). *Erm* genes are located mostly on plasmids, so probably they spread among activated sludge bacteria in HGT.

Mef genes, responsible for active antibiotic removal from the bacterial cell, are present only in two strains, Gram-positive E1 and Gram-negative E4 (Figure 2, Table 4). These elements are located in bacterial chromosome, so that could be the explanation of its rarer occurrence. Nonetheless, the resistance transfer is possible and it was previously stated that these genes can be transferred from Gram-positive cocci to Gram-negative bacteria within transposons (Brisson-Nöel et al. 1988).

Strains E1 and E4 possess not only *mef* but also *ermB* genes. Probably their resistance is caused by both mechanisms. Such a combination of resistance genes was observed previously (Bley et al. 2011) and these strains presented higher resistance against erythromycin than strains possessing two genes separately.

Regardless of the erythromycin resistance revealed with plating method, strains E3 and E7 possessed none of the two resistance genes. It suggests that their erythromycin resistance is caused by some other genes, such as: *ere*, *msr*, *mph* or other *erm* (except *erm*B) (Sutcliffe et al. 1996). In case of Gram-negative bacteria (such as *Escherichia coli*) it is possible that the resistance is based on enzymatic modification of antibiotic by *ere* coded esterases (Sutcliffe et al. 1996). PCR amplification of *mef* and *ermB* gave convergent results with MIC values. Strains with *ermB* gene present higher MIC (above 1024 μg mL^{-1}), while the MIC values for other strains without *erm*B (E3 and E7) are lower. As it was previously stated, *erm* encoded resistance is linked with MIC values higher than these presented by strains possessing *mph* or *mef* encoded resistance (Martin et al. 2001, Nguyen et al. 2009). It could be suspected that the 23S rRNA methylation is much more effective as an erythromycin resistance mechanism than the mechanism presented by strains E3 and E7. Nonetheless, the resistance paths for these isolates should be investigated.

Among nine sufamethoxazole/trimethoprim isolates only one strain was Gram-positive. MIC values pointed that all the strains were highly resistant to sulfamethoxazole/trimethoprim (MIC > 1024 μg mL^{-1}) (Table 4). In case of *dhfr*/*sul*

genes combination it is justified, but in case of strain B7, which does not possess any of the investigated genetic determinant, such high resistance can be caused by chromosomal mechanisms of the resistance (Huovinen et al. 1995).

All strains, as well as the activated sludge samples, underwent PCR amplification with *dhfrA1* and *dhfrA14* primers (flanking the most commonly present trimethoprim resistance genes) and *sul1*, *sul2* and *sul3* primers (targeting sulfamethoxazole resistance genes). Such primer sets were chosen on the basis of the previous research. It has been previously stated (Brolund et al. 2010) that *dhfr* genes were spread among *E. coli* (96% of the isolates) and *Klebsiella pneumoniae* strains (68% of the isolates, with the most often appearing *dhfrA1* gene). For *E. coli* second the most often appearing resistance gene was *dhfrA14*. Also, it has been reported previously (Dworniczek et al. 2007) that the *dhfrA1* gene was the most commonly present in clinical *E. coli* strains. The most common trimethoprim resistance gene for *Acinetobacter* spp. and *Campylobacter* spp. was *dhfrA1*. Both genes: *dhfrA1* and *dhfrA14* were present in strains B5 and B9. But in five strains none of the *dhfr* genes studied was present. According to the literature (Brolund et al. 2010) there are over 30 types of *dhfr* genes, so there is a high probability that these strains possess one of the other trimethoprim resistance coding genes.

The amplification of *sul1, sul2* and *sul3* genes revealed (Figure 3) that *sul1* was present in the activated sludge sample and all analyzed strains except B7 and B8. In case of *sul2,* positive amplification was obtained for four strains: B3, B4, B5, B9 and the activated sludge sample. No *sul3* gene amplification was obtained. In this experiment *sul1* seems to be more frequent among the activated sludge isolates than *sul2*. These results are confirmed by previous works. The research (Toleman et al. 2007) performed on *Stenotrophomonas maltophilia* and in that case 17/25 strains possessed *sul1* gene. The further research (Hoa et al. 2008) confirmed that data. In the case of previous research (Sköld 2001, Perreten and Boerlin 2003) *sul1* and *sul2* appear with an equal frequency among Gram-negative clinical strains. The absence of *sul3* in the investigated activated sludge and strains is not odd. This gene is rare, it is present only among 30% *E. coli* isolated in research performed in 2003 (Perreten and Boerlin 2003). For clinical isolates of *E. coli* only 3% of bacteria possessed *sul3*, and in wastewater analysis the frequency of *sul3* presence was ca. 14% (Grape et al. 2003). The coexistence of *sul1* and *sul2* was stated for strains B3, B4, B5, B9 and activated sludge samples as it has been previously stated (Hoek et al. 2005). For strains B7 and B8 no *sul* was present. Due to the fact that *sul* genes are the only known sulfamethoxzole resistance genes, it could be suspected that for these strains, their resistance is caused by DHPS (enzyme) or PABA (substrate – para-aminobenzoic acid) overproduction. For B5 and B9 all investigated genetic determinants of the analyzed resistance except *sul3* (absent in all cases) were present.

It was stated that there is over 140 already investigated plasmids carrying antibiotic and other toxic compounds resistance genes (Rahube and Yost 2010), so it is essential to monitor permanently their presence in WWTPs. The possibility that these genetic elements can be directed to water tracts outside WWTPs has been found to be a serious public health risk. In this experiment it was stated that the PCR-based method can be useful for erythromycin and sulfamethoxazole/trimethoprim resistance monitoring in WWTP activated sludge biocenosis. The results obtained for pure strains and activated sludge samples are convergent. Together with analytical method (HPLC), microbial plating and DGGE as a tool for biocenosis genetic structure studies, it is possible to estimate: the presence of resistance genes, the influence of antibiotic presence in wastewater to gaining resistance against them and the direction of microbial community changes linked with bacterial changeability.

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Wykrywanie genów oporności na antybiotyki w oczyszczalni ścieków – podejście klasyczne i biologii molekularnej

Streszczenie: Antybiotyki to grupa związków potencjalnie szkodliwych dla środowiska. Odgrywają one rolę w procesach transferu antybiotykooporności pomiędzy patogenami i bakteriami niechorobotwórczymi. Wykorzystując metodę wysokosprawnej chromatografii cieczowej (HPLC) wykazano obecność erytromycyny, sulfametoksazolu i trimetoprimu w miejskiej oczyszczalni ścieków w następujących stężeniach: dla erytromycyny < 20 ng L^{-1}, N-acetylo-sulfametoksazolu 3349 ± 719 i 2933 ± 429 ng L^{-1}, a trimetoprimu 400 ± 22 i 364 ± 60 ng L^{-1}, odpowiednio: zimą i latem. Ponieważ antybiotykooporność bakteryjna może być stymulowana obecnością antybiotyków w środowisku, istnieje możliwość pojawienia się wielu szlaków opornościowych u bakterii narażonych na działanie tych związków. Dlatego też podjęto próbę detekcji wybranych genów oporności na badane chemioterapeutyki metodą łańcuchowej reakcji polimerazy (PCR). Obecność elementów genetycznych badano zarówno w szczepach bakteryjnych, u których udowodniono oporność na badany związek bakteriobójczy, jak i w próbce osadu czynnego, z którego te bakterie izolowano. Do badań wybrano najczęściej występujące geny oporności: dla erytromycyny *erm* i *mef*, dla sulfametoksazolu: *sul1*, *sul2*, *sul3*, a dla trimetoprimu *dhfrA1* i *dhfr14*. Wykazano, że wartość minimalnego stężenia inhibitującego (MIC), nie koresponduje z obecnością większej liczby mechanizmów oporności. Większość szczepów opornych wykazywała tylko jeden z badanych mechanizmów oporności na antybiotyk niezależnie od wartości MIC. Potwierdzono również możliwość monitorowania obecności genów oporności na antybiotyki metodą PCR bezpośrednio w osadzie czynnym. Ze względu na ograniczona liczbę izolatów użytych w tym eksperymencie wyniki uzyskane w pracy powinny być traktowane jako wstęp do dalszych badań.