Monitoring of drug resistance amplification and attenuation with the use of tetracycline-resistant bacteria during wastewater treatment

Monika Harnisz[1,*], *Ewa* Korzeniewska[1], *Sebastian* Niestępski[1], *Adriana* Osińska[1], and *Beata* Nalepa[2]

[1]Department of Environmental Microbiology, University of Warmia and Mazury in Olsztyn, ul. Prawocheńskiego 1, 10-957 Olsztyn, Poland
[2]Department of Food and Industrial Microbiology, University of Warmia and Mazury, Plac Cieszyński 1, 10-726 Olsztyn, Poland

Abstract. The objective of this study was to monitor changes (amplification or attenuation) in antibiotic resistance during wastewater treatment based on the ecology of tetracycline-resistant bacteria. The untreated and treated wastewater were collected in four seasons. Number of tetracycline- (TET^R) and oxytetracycline-resistant (OTC^R) bacteria, their qualitative composition, minimum inhibitory concentrations (MICs), sensitivity to other antibiotics, and the presence of *tet*(A, B, C, D, E) resistance genes were determined. TET^R and OTC^R counts in untreated wastewater were 100 to 1000 higher than in treated effluent. OTC^R bacterial counts were higher than TET^R populations in both untreated and treated wastewater. TET^R isolates were not dominated by a single bacterial genus or species, whereas *Aeromonas hydrophila* and *Aeromonas sobria* were the most common in OTC^R isolates. The treatment process attenuated the drug resistance of TET^R bacteria and amplified the resistance of OTC^R bacteria. In both microbial groups, the frequency of *tet*(A) gene increased in effluent in comparison with untreated wastewater. Our results also indicate that treated wastewater is a reservoir of multiple drug-resistant bacteria as well as resistance determinants which may pose a health hazard for humans and animals when released to the natural environment.

1 Introduction

Microbial resistance to drugs poses a growing threat for human health and life. The wide-spread use of antibiotics in the treatment of bacterial diseases has contributed to the development of strains that are resistant to this group of drugs. The problem has been escalating dramatically by the increased use of antimicrobial agents in medicine, detergent production, agriculture, breeding and cosmetic industry [1–3]. Multiple drug resistance has developed and spread rapidly among various bacterial groups within a short timeline of

* Corresponding author: monika.harnisz@uwm.edu.pl

© The Authors, published by EDP Sciences. This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (http://creativecommons.org/licenses/by/4.0/).

evolution. The problem is deepened by the existence of large bacterial populations, short generation times and effective mechanisms of genetic information exchange [4].

Wastewater treatment plants (WWTPs) are flagship projects aiming to prevent environmental pollution. Wastewater purified of suspended matter, excess organic substances and biogenic elements can be reused to irrigate fields or evacuated to surface waters [6, 7]. Municipal treatment plants are supplied with wastewater generated by households, hospitals, public utility buildings, industrial plants as well as rain water. This combination of organic substances, biogenic elements and trace amounts of antibiotics supports the development and survival of drug-resistant bacteria [8]. The use of activated sludge and biological membrane systems in the treatment process contributes to the exchange of genetic information between bacteria of the same or different species, including microorganisms from unrelated families [9]. The above facilitates the exchange of genes located on mobile plasmids, including those encoding drug resistance [10].

WWTPs are important reservoirs of antibiotic resistance of both commensal and pathogenic organisms [11, 12]. Drug-resistant bacteria and resistance genes can be released from the plant into the natural environment. To mitigate this threat, the presence of antibiotic-resistant bacteria has to be determined in untreated and treated wastewater. The resulting data support an evaluation of the effect of the treatment process on changes in bacterial resistance to antibiotics.

The microbial indicator has been defined by Nielsen et al. [13] as “microbial parameter that represents properties of the environment or impacts to the environment, which can be interpreted beyond the information that the measured or observed parameter represents itself”. Microbial bioindicators could be based on functional or structural diversity of the community [14]. In the case of changes in the antibiotic resistance of bacteria, structural bioindicators are specific bacterial species and genera which are cultured on selective media and functional bioindicators comprise groups of bacteria resistant to a specific drug or a class of drugs. They are grown on nutrient agars, such as TSA, with the addition of antibiotics, such as tetracyclines which, despite a rapid increase in bacterial resistance, continue to be widely used in human and veterinary medicine.

The objective of this study was to monitor changes (amplification or attenuation) in antibiotic resistance during wastewater treatment based on the ecology of tetracycline- and oxyteracycline-resistant bacteria in untreated and treated wastewater. In Poland, tetracycline is one of the most popular drug of choice in human medicine, whereas oxytetracycline is widely used in the treatment of both humans and animals. Akinbowale et al. [15] noticed, that minimum inhibitory concentrations of these two drugs can be different, so we thought the populations of tetracycline- and oxytetracycline-resistant bacteria can be different, also. Therefore, we decided to study both microbial groups.

2 Materials and Methods

2.1. Wastewater treatment plant

The investigated site was the Łyna Municipal Waste Treatment Plant in Olsztyn. The plant's process line comprises mechanical, biological and chemical treatment sections, as well as sludge processing units. The plant has the following technical specification: treatment system - activated sludge, average processing capacity – 60,000 m^3/d, wastewater type - municipal wastewater, mechanical treatment devices – screenings, grit chamber and pre-sedimentation tank, biological treatment devices – separation chambers, aeration chambers and secondary sedimentation tanks, sedimentation devices – closed and open digestion chambers, belt filter press, incinerator.

2.2. Sample collection

The experimental material comprised samples of untreated (from grid chamber) and treated (effluent of secondary sedimentation tank) wastewater collected on four occasions: in April, July and October 2012 and in February 2013. Wastewater samples were collected into sterile bottles, transported to the laboratory at a temperature of 4°C and processed on the day of collection.

2.3. Heterotrophic plate counts and counts of tetracycline- and oxytetracycline-resistant bacteria

Heterotrophic plate counts (HPC), counts of tetracycline-resistant bacteria (TET^R) and counts of oxytetracycline-resistant bacteria were determined in plates containing the TSA medium (Oxoid) without/with tetracycline and oxytetracycline supplementation, respectively. The plates were cultured at a temperature of 30°C for 24h. According to CLSI guidelines [16], pathogens with MIC values of the tested antibiotics at ≥ 16 μg/mL are regarded as resistant. In this study, tetracycline and oxytetracycline were applied at a concentration of 32 μg/mL to isolate highly resistant bacteria. MIC value of these antibiotics were determined by the agar dilution method with final antibiotic concentrations in the range of 32 to 512 μg/mL. Cultured colonies of HPC, TET^R and OTC^R were counted, and the results were stated in terms of colony forming units (CFU) per mL of wastewater. Colonies with various phenotypes were isolated from the TSA medium supplemented with tetracycline and oxytetracycline. A total of 40 isolates were obtained from each medium (in summary 80 isolates). The strains were stored on the LB medium (Merck) with 10% glycerol at -70°C for further analyses.

2.4. Antibiotic susceptibility testing

TET^R and OTC^R strains isolated in the study were subjected to sensitivity tests against nine antimicrobials from five classes: (1) beta-lactams: mezlocillin (MEZ 75 μg), ampicillin (AMP 10 μg), piperacillin (PRL 75 μg); amoxicillin/clavulanic acid (AMC 20/10 μg), ceftazidime (CAZ 30 μg), cefotaxime (CTX 30 μg); (2) aminoglycosides: gentamicin (CN 10 μg), tobramycin (TOB 10 μg); (3) macrolides: erythromycin (TOB 10 μg); (2) tetracyclines: tigecycline (TGC 15 μg); (4) trimethoprim/sulfamethoxazole (SXT 1.25/23.75 μg); (6) phenicols: chloramphenicol (C 30 μg); and (6) fluoroquinolones: ciprofloxacin (CIP 30 μg). All disks were supplied by Oxoid. Resistance was estimated by measuring the inhibition zone according to the guidelines of CLSI [16].

2.5. Identification of isolates and multiplex PCR of tetracycline-resistant genes

Genomic DNA was isolated by the use of thermal lysis [14]. TET^R and OTC^R isolates were identified by 16S rRNA gene sequencing in accordance with a previously described method [14].

The mechanism of molecular resistance to tetracyclines was analyzed with the use of five out of around 40 known determinants [17]. They were selected based on source data indicating that *tet*(A), (B), (C), (D) and (E) genes are the most popular determinants of resistance in *Enterobacteriaceae* and *Aeromonas* sp. bacteria [18, 19]. Multiplex PCR was conducted in line with the methodology proposed by Nawaz et al. [18]. For all reactions, standard PCR mixtures without DNA template were used as negative controls. Plasmids

carrying *tet* genes or the 16S rRNA gene verified by sequencing were used as positive controls.

2.6. Data analyses

One way analysis of variance (ANOVA, StatSoft Inc., 1984–2017) was used to check differences in the abundance of the studied bacterial groups between the analyzed sites.

3 Results

HPC analyses revealed differences in the size of TET^R and OTC^R bacterial populations, subject to the site and time of sampling. TET^R and OTC^R concentrations in untreated wastewater were 100 to 1000-fold higher than in discharged effluent ($p = 0.006$ and $p = 0.003$ for TET^R and OTC^R, respectively) (Table 1).

Table 1. The share of tetracycline- and oxytetracycline-resistant strains incubated at a temperature of 30°C (TET^R30°C and OTC^R30°C) in total heterotrophic bacterial counts incubated under identical conditions (HPC30°C).

Bacterial group		Date	Sampling site	
			Untreated wastewater	Treated wastewater
TET^R 30°C [1)]	CFU/cm^3	04.2012	6.18×10^4	8.40×10^2
		07.2012	1.08×10^5	1.26×10^3
		10.2012	1.15×10^5	3.80×10^2
		02.2013	3.36×10^4	7.02×10^2
OTC^R 30°C [2)]		04.2012	3.51×10^5	6.61×10^2
		07.2012	1.23×10^5	1.52×10^3
		10.2012	1.99×10^5	1.86×10^3
		02.2013	2.74×10^5	6.50×10^3
HPC 30°C [3)]		04.2012	5.01×10^6	3.67×10^4
		07.2012	9.70×10^6	7.40×10^4
		10.2012	9.60×10^6	2.05×10^4
		02.2013	4.88×10^6	1.21×10^5
Share of TET^R 30°C in HPC 30°C	%	04.2012	1.23	2.3
		07.2012	1.1	1.7
		10.2012	1.2	1.85
		02.2013	0.7	0.6
Share of OTC^R 30°C in HPC 30°C		04.2012	7	1.8
		07.2012	1.3	2.1
		10.2012	2.1	9.1
		02.2013	5.6	5.4

[1)] – tetracycline-resistant bacteria cultured at 30°C after 2 days of incubation;
[2)] – oxytetracycline-resistant bacteria cultured at 30°C after 2 days of incubation;
[3)] – total heterotrophic bacterial counts determined at 30°C after 2 days of incubation;

Maximum TET^R levels were noted in October and July 2012 in untreated and treated wastewater, respectively. The highest OTCR counts in inflows and outflows of WWTP were reported in April 2012 and February 2013. OTCR bacteria were more abundant than TETR bacteria in both untreated and treated wastewater samples ($p = 0.012$). In both incoming and outgoing wastewater, TETR had the lowest share of HPC in February 2013, and the highest – in April 2012. OTC^R was characterized by a different share of HPC. The percentage share of OTC^R bacteria in untreated sewage was the lowest in July 2012 and the

highest in April 2012. As regards treated wastewater, the lowest and the highest percentages of OTC^R bacteria were noted in April 2012 and October 2013, respectively.

In TET^R bacteria, the predominance of a single genus or species was not observed. In the group of 40 OTC^R isolates, eight *Aeromonas hydrophila*, ten *Aeromonas sobria*, one *Aeromonas jandei* and two *Aeromonas allosacharphila* strains were identified and found to predominate (Table 2).

In the group of TET^R bacteria isolated from untreated wastewater, *Acinetobacter baumanii* was characterized by very low sensitivity to drugs (MIC of TET – 256 µg/mL, MIC of OTC – 512 µg/mL, MDR to seven groups of drugs). The least drug-sensitive microorganism isolated from the OTC^R group in untreated wastewater was *Aeromonas sobria* (MIC of TET – 32 µg/mL, MIC of OTC – 256 µg/mL, resistant to five classes of UW - untreated wastewater, TW - treated wastewater; MDR to: B – β-lactams, A-Aminoglycosides, M-Macrolides, T-Tetracyclines, S-Sufonamides/Trimethoprim, P-Phenicols, F-Fluoroquinolones; nd - not detected; - resistance to less than three classes of drugs antibiotics). The most frequently observed pattern of resistance was insensitive to β-lactams, aminoglycosides and macrolides (Table 2).

Table 2. Diversity of tetracycline- and oxytetracycline-resistant bacteria, minimum inhibitory concentrations of tetracycline (TET) and oxytetracycline (OTC), the resistance profile, multi-drug resistance (MDR) and determinants of resistance of strains isolated from UW and WW.

Origin		Identification results (number of strains)	MIC of TET/OTC	Resistance profile (number of strains)	MDR	Genes
UW	TET^R	*Acinetobacter baumanii(2)*	256/512	MEZ, CAZ, CTX, CN, TOB, E,SXT,C,CIP(1) AMP,AMC,PRL,CAZ,CTX,E,TGC,C(1)	BAMTSPF BMTP	*tet*(B) *tet*(B)
		Acinetobacter junii(2)	96/512	MEZ,AMP,AMC,PRL,CAZ,CTX,E,TGC,C(1) AMP,PRL,CAZ,CTX,E,TGC,C(1)	BMTP BMTP	*tet*(A),*tet*(B) *tet*(B)
		Burkholderia cepacia(3)	96/128	AMP(1) AMP,PRL,CAZ,CTX,E,TGC,C(1) AMP,AMC,PRL,CAZ,CTX,E,TGC,C(1)	- BMTP BMTP	*tet*(A) *tet*(A) *tet*(A)
		Klebsiella pneumoniae(1)	96/512	AMP,E,TGC(1)	BTP	*tet*(E)
		Pasteurella pneumotropica(1)	32/128	MEZ,AMP,AMC,PRL,CAZ,CTX,E,TGC,C(1)	BMTP	nd
		Plesiomonas shigelloides(3)	96/128	MEZ,AMP,AMC,CAZ,CTX(1) MEZ,AMP,AMC,PRL,CAZ,CTX,E,TGC,C(2)	- BMTP	*tet*(E) *tet*(E)
		Pseudomonas putida(1)	64/128	MEZ,AMP,AMC,PRL,CAZ,CTX,E,TGC,C	BMTP	*tet*(D)
		Salmonella arizonae(3)	128/512	AMP,CTX(1) MEZ,AMP,AMC,PRL,CAZ,CTX,E,TGC,C(1) AMP,AMC,PRL,CAZ,CTX,E,TGC,C(1)	- BMTP BMTP	*tet*(D) *tet*(D) *tet*(D)
		Serratia marcescens(3)	128/512	MEZ,AMP,AMC,CAZ,CTX,CIP(1) MEZ,AMP,AMC,PRL,CAZ,CTX,E,TGC(1) AMP,AMC,PRL,CAZ,CTX,E,TGC,C(1)	- BMT BMTP	*tet*(D) *tet*(D) *tet*(D)
		Sphingomonas multivorum(1)	64/512	AMP,PRL,CAZ,CTX,E,TGC,C(1)	BMTP	nd
	OTC^R	*Aeromonas hydrophila(4)*	128/256	AMP,E(2) AMP,AMC,E(2)	- -	*tet(A)* *tet(B)*
		Aeromonas sobria(6)	32/128 32/256 32/128 32/128	AMP,E(1) AMP,SXT,C,CIP(2) AMP,CAZ,CTX,CN,TOB,E,SXT,C,CIP(2) AMP,MEZ,CAZ,CTX,CN,TOB,E(1)	- - BAMSP BAM	*nd* *tet(A),tet(B)* *tet(A),tet(B)* *nd*
		Citrobacter	128/128	MEZ,AMP,AMC,CAZ,CTX(1)	-	*tet(A)*

		freundii(2)	96/128	MEZ,CAZ,CTX,CN,TOB,E(1)	BAM	*tet(A)*
		Escherichia coli(3)	96/256	MEZ,AMP,AMC,CAZ,CTX(1) MEZ,CAZ,CTX,CN,TOB,E(2)	- BAM	*tet(A)* *tet(A)*
		Pseudomonas putida(1)	96/128	SXT,C,CIP(1)	SPF	*tet(A)*
		Sphingomonas multivorum(2)	32/128	AMP,CTX(1) MEZ,CAZ,CTX,CN,TOB,E(1)	- BAM	*tet(A)* *tet(A)*
		Yersinia pestis(2)	32/128	MEZ,AMP,AMC,CAZ,CTX(1) MEZ,CAZ,CTX,CN,TOB,E(1)	- BAM	*tet(E)* *tet(E)*
TW	TET^R	*Acinetobacter baumanni(2)*	32/256	MEZ,AMP,PRL,E,CIP(2)	BMF	*tet*(A)
		Acinetobacter junii(2)	32/512	MEZ,AMP,PRL,E,CIP(1) MEZ,AMP,PRL,E,SXT,CIP(1)	BMF BMF	*tet*(A) *tet*(A)
		Klebsiella pneumoniae(3)	32/256 128/256	AMP,AMC(2) MEZ,AMP,AMC,CAZ,CTX(1)	- -	*tet*(E) *tet*(E)
		Pasteurella pneumotropica(3)	32/256 128/256	MEZ,AMP,PRL,E,SXT,CIP(2) MEZ,AMP,AMC,CAZ,CTX(1)	BMSF -	*tet*(A) *tet*(A)
		Pseudomonas putida(3)	32/512 32/512 128/512	AMP,E(1) CTX,E(1) MEZ,AMP,PRL,E(1)	- - -	*tet*(A) *tet*(A) *tet*(A)
		Salmonella arizonae(2)	32/512 128/512	MEZ,AMP,CTX,E(1) MEZ,AMP,PRL,E(1)	- -	*tet*(E) *tet*(E)
		Serratia marcescens(3)	32/128 256/512	MEZ,AMP,AMC,PRL,E,SXT,CIP(1) E,CIP(2)	BMSF -	*tet*(A),*tet*(B) nd
		Vibrio parahaemolyticus(2)	32/128	MEZ,AMP,PRL,CAZ,CTX(1) MEZ,AMP,AMC,PRL,E,SXT,CIP(1)	- BMSPF	*tet*(E) *tet*(E)
	OTC^R	*Aeromonas allosaccharophila(2)*	32/512	MEZ,AMP,AMC,PRL,CTX,E,TGC,SXT,C(1) MEZ,AMP,PRL,E,TGC,SXT(1)	BMTSP BMTS	*tet*(A) *tet*(A)
		Aeromonas hydrophila(4)	128/512	AMP,E(1) AMP,AMC,E(1) AMP,AMC,E(1) AMP,AMC,E(1)	- - - -	*tet*(A) *tet*(A) *tet*(B) *tet*(E)
		Aeromonas jandei(1)	32/512	MEZ,AMP,AMC,PRL,CTX,E,TGC,C(1)	BMTP	*tet*(A)
		Aeromonas sobria(4)	128/512(4)	MEZ,CAZ,CTX,CN,TOB,E,SXT,C,CIP(4)	BAMSPF(4)	*tet*(A)(2) *tet*(B) -
		Citrobacter freundii(1)	128/256	MEZ,AMP,AMC,CAZ,CTX,E,SXT(1)	BMS	*tet*(A)
		Flavimonas oryzihabitans(1)	128/128	PRL,CAZ,TOB,E(1)	BAM	*tet*(A)
		Pantoea spp.(1)	96/128	MEZ,AMP,AMC,CAZ,CTX(1)	-	*tet*(D)
		Pseudomonas putida(1)	128/512	AMP,AMC,CAZ,TOB,E,TGC,C(1)	BAMTP	*tet*(A)
		Salmonella spp.(2)	32/96	MEZ,AMP,PRL,E,CIP(2)	BMF	*tet*(E)
		Shewanella putrefaciens(2)	32/96	AMP,AMC,CAZ,E(2)	-	*tet*(A),*tet*(B)
		Vibrio metschnikovii(1)	32/96	MEZ,AMP,PRL,CAZ,CTX(1)	-	*tet*(A)

In treated wastewater samples, TET^R group isolates which were the least sensitive to the tested drugs were *Vibrio parahaemoliticus*, *Klebsiella pneumoniae* and *Pasteurella pneumotropica* (MIC of TET – 32 μg/mL, MIC of OTC – 128 or 256 μg/mL, resistant to four or five classes of antibiotics). In the OTC^R group, the highest drug resistance was reported for *Aeromonas sobria* (MIC of TET – 128 μg/mL, MIC of OTC – 512 μg/mL,

MDR to six classes of drugs). The isolates from treated wastewater were characterized by multiple drug resistance. In the TET^R group 45% of isolates were multidrug resistant to three or four groups of antibiotics, in the OTC^R group 60% isolates were multidrug bacteria (Table 2). Regardless of the sampling site, the MIC values of tetracycline were always lower than oxytetracycline MICs (Table 2). In both types of analyzed wastewater, tetracycline MICs were determined in the range of 32 to 256 µg/mL. The MIC values of OTC ranged from 96 to 512 µg/mL in untreated sewage, and from 128 to 512 µg/mL in treated wastewater. In treated wastewater samples, the majority of TET^R and OTC^R isolates were characterized by the presence of the *tet*(A) gene which was noted in 60% strains. In the group of TET^R strains showing the lowest levels of drug resistance, the presence of the *tet*(A) and *tet*(B)gene was noted (*Vibrio parahaemoliticus* and *Klebsiella pneumoniae*). In treated wastewater, OTC^R strains of the genus *Aeromonas* featured *tet*(A), *tet*(B) and *tet*(E) genes (Table 2).

4 Discussion

The treatment process decreased TET^R and OTC^R bacterial counts by two orders of magnitude. The abundance of OTC^R bacteria was somewhat higher in both untreated and treated wastewater samples. There is a general scarcity of data regarding the populations of wastewater-borne heterotrophic bacteria resistant to tetracycline and oxytetracycline. In studies of treated wastewater, Kim et al. [20] and Huang et al. [11] determined TET^R counts in the range of 10^2 - 10^3 CFU/mL, and their findings are consistent with our results. Vast similarities are also observed in TET^R bacteria's share of HPC which was determined at 0 to 2% by Kim et al. [20], Huang et al. [11] and Munir et al. [21] and at 1.61% in this study. It should be noted, however, that tetracycline concentrations reached 16 µg/ml in the experiment described by Huang et al. [11], compared with 32 µg/mL in a study by Kim et al. [20] and in the present study. Then, bacteria resistant to tetracyclines constitute a small part of HPC in treated wastewater - it may be due to of lack natural resiatance to tetracyclines among bacteria. Tet^R isolates were not marked by a predominance of a single bacterial species or genus, whereas bacteria of the genus *Aeromonas* were the most abundant microorganisms in the group of OTC^R isolates. *Aeromonas* bacteria are popularly observed in both untreated wastewater [22] and treated effluent from fish farms [23, 24]. Huys et al. [25] and Rhodes et al. [26] found more than 85% bacteria of the genus *Aeromonas* to be oxytetracycline-resistant. In this study, relatively large populations of *Enterobacteriaceae* microorganisms were reported in both, TET^R and OTC^R isolates, and similar observations were made by Guillaume et al. [27] and Moura et al. [28]. A comparison of MIC values and multiple drug resistance of the least resistant TET^R isolates in samples of untreated and treated wastewater indicates a drop in resistance to tetracycline, oxytetracycline and a decrease in multiple drug resistance as a result of sewage treatment. The reverse was observed in the OTC^R group which resistance to tetracycline and oxytetracycline decreased with an increase in the number of multiple drug-resistant strains. In the TET^R group, the majority of isolates obtained from untreated wastewater were resistant to four classes of antibiotics, whereas bacterial isolates from treated wastewater samples were resistant to one or two groups of antibiotics. The majority of OTC^R bacteria identified in untreated wastewater were not multidrug resistant, however four isolates found in treated effluent samples were resistant to of all analyzed antibiotics. Our findings are consistent with the results of published studies [29]. The results of our previous study [30] and other authors' findings [31, 32] testify to the high drug resistance of TET^R and OTC^R bacteria. According to Hall et al. [33], resistance to a single antibiotic could lead to selective resistance to other drugs and, ultimately, multiple-drug resistance. In untreated wastewater isolates, there was not the predominant gene in the TET^R group, *tet*(A) was

most frequently determined in the OTC^R groups, whereas the predominance of *tet*(A) gene was noted in both bacterial groups from treated effluent. Sandalli et al. [34] and Tao et al. [19] have demonstrated the predominance of the *tet*(A) gene in *Enterobacteriaceae* bacteria, and Harnisz et al. [14] - in bacteria of *Aeromonas* genus. The *tet*(A) gene, which encodes the removal of tetracyclines from the cell with the involvement of transport proteins, is often found on plasmids, including conjugating plasmids [35]. In this study, the high frequency of *tet*(A) noted in untreated and treated wastewater, could be attributed to the gene's presence on mobile plasmids. In the work of Guillaume et al. [27], *tet*(A) was found to be highly stable in *Salmonella* sp. isolated from treated wastewater. The presence of *tet*(B), *tet*(E) and, rarely, *tet*(D) determinants has also been noted in TET^R and OTC^R isolates, whereas the *te*t(C) gene was not found. Similar results were reported by Börjesson et al. [36], Sandalli et al. [34] and Tao et al. [19]. Auerbach et al. [37] observed a wide variety of tetracycline resistance genes in wastewater, including *tet*(C) which was not identified in this study. The above could attest to the "regional" occurrence of tetracycline resistance determinants, subject to the climate, the intestinal microbiota of the local population and various antibiotic treatments. In this experiment, the simultaneous presence of two resistance determinants, *tet*(A) and *tet*(B), was noted only in two strains of the family *Enterobacteriaceae*: *Serratia marcescens* and *Shewanella putrefaciens* and three isolates of genus *Acinteobacter* and *Aeromonas*. According to Tao et al. [19], around 30% bacteria have at least two genes encoding tetracycline resistance. Similar findings were reported in studies of other bacterial species by Akinbowale et al. [15], Henriques et al. [31] and Nikolakopoulou et al. [32].

5 Conslusions

Based on the results of this study, it can be concluded that the drug resistance of TET^R bacteria was attenuated and the drug resistance of OTC^R bacteria was amplified in the course of the treatment processes. In both bacterial groups, the frequency of the *tet*(A) gene was high in untreated and treated wastewater. Our results also indicate that treated wastewater is a reservoir of multiple drug-resistant bacteria as well as resistance determinants which may pose a health hazard for humans and animals when released to the natural environment.

References

1. F. Baquero, J.L. Martinez, R. Canton, Curr. Opin. Biotech. **19**, 260–265 (2008)
2. J. Williams-Nguyen, J.B. Sallach, S. Bartelt-Hunt, A.B. Boxall, L.M. Durso, J.E. McLain, R.S. Singer, D.D. Snow, J.L. Zilles, J. Environ. Qual. **45**, 394–406 (2016)
3. K.S. Le Corre, C. Ort, D. Kateley, B. Allen, B.I. Escher, J. Keller, Environ. Int. **45**, 99–111 (2012)
4. L. Boto, Proc. R. Soc. B. **277**, 819–827 (2010)
5. Clinical and Laboratory Standards Institute, Performance standards for susceptibility testing, M100–S15. CLSI, Wayne, Pennsylvania, USA (2005)
6. E. Korzeniewska, M. Harnisz, J. Environ. Manage. **128**, 904–911 (2013)
7. M.L. Luprano, M. De Sanctis, G. Del Moro, C. Di Iaconi, A. Lopez, C. Levantesi, Sci. Total Environ. **571**, 809–818 (2016)
8. A. Schlüter, R. Szczepanowski, A. Pühler, FEMS Microbiol. Rev. **31**, 449–477 (2007)
9. R. Szczepanowski, B. Linke, I. Krahn, Microbiol. **155**, 7, 2306–2319 (2009)

10. A. Osińska, M. Harnisz, E. Korzeniewska, Environ. Sci. Pollut. Res. **23**, 10818–10831 (2016)
11. J.J. Huang, H.Y. Hu, S.Q. Lu, Y. Li, F. Tang, Y. Lu, B. Wei, Environ. Int. **42**, 31–36 (2011)
12. A. Osińska, E. Korzeniewska, M. Harnisz, S. Niestępski, Sci. Total Environ. **577**, 367–375 (2017)
13. M.N. Nielsen, A. Winding, Technical Report 388, National Environmental Research Institute, Denmark (2002)
14. M. Harnisz, E. Korzeniewska, S. Ciesielski, I. Gołaś, Sci. Total Environ. **505**, 704–711 (2015)
15. A.L. Akinbowale, H. Peng, M.D. Barton, J. Appl. Microbiol. **100**, 1103–1113 (2006)
16. Clinical and Laboratory Standards Institute (CLSI). Performance standards for susceptibility testing, M100–S15. Wayne, Pennsylvania, USA (2014)
17. Chopra, M. Roberts, Microbiol. Mol. Biol. R. **65**, 232–260 (2001)
18. M. Nawaz, K. Sung, S.A. Khan, A.A. Khan, R. Steele, Appl. Environ. Microbiol. **72**, 6461–6466 (2006)
19. R. Tao, G.G. Ying, H.C. Su, H.W. Zhou, J.P.S Sidhu, Environ. Pollut. **158**, 2101–2109 (2010)
20. S. Kim, H. Park, K. Chandran, Chemosphere **78**, 1071–1077 (2010)
21. M. Munir, K. Wong, I. Xagoraraki, Water Res. **45**, 681–693 (2011)
22. V. Figueira, I. Vaz-Moreira, M. Silva, C.M. Manaia, Water Res. **45**, 5599–5611 (2011)
23. M. Harnisz, S. Tucholski, Ecol. Eng. **36**, 4, 466–470 (2010)
24. M. Harnisz, E. Korzeniewska, I. Gołaś, Chemosphere **128**, 134–141 (2015)
25. G. Huys, D. Gevers, R. Temmerman, M. Cnockaert, R. Denys, G. Rhodes, R. Pickup, P. McGann , M. Hiney, P. Smith, J. Swings, J. Syst. Appl. Microbiol. **24**, 1, 122–130 (2001)
26. G. Rhodes, G. Huys, J. Swings, P. McGann, M. Hiney, P. Smith, J. Swings, Appl. Environ. Microbiol. **66**, 3883–3890 (2000)
27. G. Guillaume, D. Verbrugge, M.L. Chasseur-Libotte, W. Moens, J.M. Collard, FEMS Microbiol. Ecol. **32**, 77–85 (2000)
28. A. Moura, I. Henriques, R. Ribeiro, A. Correia, J. Antimicrob. Chemoth. **60**, 1243–1250 (2007)
29. Y. Zhang, C.F. Marrs, C. Simon, C. Xi, Sci. Total Environ. **407**, 3702–3706 (2009)
30. M. Harnisz, I. Gołaś, M. Pietruk, Ecol. Indic. **11**, 663–668 (2011)
31. I.S. Henriques, F. Fonseca, A. Alves, M.J. Saavedra, A. Correia, Lett. Appl. Microbiol. **47**, 526–533 (2008)
32. T.L. Nikolakopoulou, E.P. Giannoutsou, A.A. Karabatsou, A.D. Karagouni, J. Microbiol. **46**, 633–640 (2008)
33. A.R. Hall, D.C. Angst, K.T. Schiessl, M. Ackermann, Evol. Appl. **8**, 261–272 (2015)
34. C. Sandalli, O.B. Ozgumus, A. Sevim, World J. Microbiol. Biot. **26**, 2099–2103 (2010)
35. A.S. Schmidt, M.S. Bruun, I. Dalsgaard, J.L. Larsen, Appl. Environ. Microb. **67**, 5675–5682 (2001)
36. S. Börjesson, A. Mattsson, P.E. Lindgren, J. Water Health **8**, 2, 247–256 (2010)
37. E.A. Auerbach, E.E. Seyfried, K.D. McMahon, Water Res. **41**, 5, 1143–1151 (2007)