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Quantitative Occurrence of Antibiotic Resistance Genes among Bacterial Populations from Wastewater Treatment Plants Using Activated Sludge

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Abstract: Wastewater treatment plants (WWTPs) are an important reservoir in the development of drug resistance phenomenon and they provide a potential route of antibiotic resistance gene (ARGs) dissemination in the environment. The aim of this study was to assess the role of WWTPs in the spread of ARGs. Untreated and treated wastewater samples that were collected from thirteen Polish WWTPs (applying four different modifications of activated sludge–based treatment technology) were analyzed. The quantitative occurrence of genes responsible for the resistance to beta-lactams and tetracyclines was determined using the real-time PCR method. Such genes in the DNA of both the total bacterial population and of the *E. coli* population were analyzed. Among the tested genes that are responsible for the resistance to beta-lactams and tetracyclines, bla_{OXA} and bla_{TEM} and *tet*A were dominant, respectively. This study found an insufficient reduction in the quantity of the genes that are responsible for antibiotic resistance in wastewater treatment processes. The results emphasize the need to monitor the presence of genes determining antibiotic resistance in the wastewater that is discharged from treatment plants, as they can help to identify the hazard that treated wastewater poses to public health.

Keywords: wastewater; qPCR; tetracyclines; beta-lactams; ARGs; *Escherichia coli*

1. Introduction

The occurrence and spread of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) is a serious health protection problem worldwide [1]. According to the World Health Organization, the occurrence of antibiotic resistance among bacterial populations is regarded as one of the major hazards and challenges to public health in the 21st century [2], since it has serious economic consequences as well as risks to the health and lives of both humans and animals [3]. A major problem in controlling this phenomenon is the lack of national or international legal regulations controlling the spread of ARB and ARGs in the environment [4]. The existing reports and definitions of drug resistant bacteria refer only to clinical strains, while similar characteristics for environmental strains are still missing [5]. The control of the aquatic environment appears particularly important, as it provides the main spread route of ARB and ARGs [6]. Even though the European Union (EU) Water Framework Directive contains a provision on ensuring the good quality of waters according to specific standards, it does not refer to the phenomenon of antibiotic resistance. The genes that are responsible for resistance to antibiotics are regarded as environmental pollutants [7] that are capable of spreading among bacteria in natural environments and drinking water resources [8,9]. This is due to the fact that

non-pathogenic bacterial species with antibiotic resistance genes may serve as a source of antibiotic resistance genes for pathogenic bacteria [10]. ARGs that are found among bacterial pathogens largely originate from the environment, which receives treated or untreated wastewater generated by animal farms, aquaculture, and industry [9,11].

One of the main reservoirs of ARB and ARG pollutants and the source of their spread in the natural environment [12–14] are wastewater treatment plants (WWTPs) and treated wastewater discharged to surface water bodies [5,6,15–17]. WWTPs receiving high concentrations of microbial contaminants with wastewater from hospitals, agriculture, and industry stimulate the transfer of genetic information between pathogenic and environmental microorganisms. In addition, the conditions prevailing in wastewater treatment plants, such as a high content of microorganism populations, the relative abundance of nutrients, and the presence of sub-threshold levels of antibiotic substances in wastewater [16], provide an environment favourable for the survival of ARB and the transfer of ARGs.

The increase in microbial diversity and the number of mobile genetic elements [18] and ARGs [19] is significantly affected by the activated sludge. Microorganisms forming particles of sludge are responsible for the accumulation and release of bacterial plasmids carrying ARGs [20], which facilitate the exchange of these genes between bacteria. Another factor contributing to the development of antibiotic resistance phenomenon during the wastewater treatment process is the presence of antibiotics, which, even at low concentrations, may induce genetic responses leading to adaptations and mutations among the microbial population [21]. Therefore, conventional activated sludge-based wastewater treatment methods provide ideal conditions for the transfer of these genes [22], which was confirmed by many authors [23–25]. The results of these studies show that the bacteria released with the discharged treated wastewater may be capable of active transmission of resistance genes among environmental microorganisms. Bengtsson-Palme et al. [26] demonstrated that the number of bacteria decreased following the wastewater treatment processes, while the abundance and diversity of genes determining antibiotic resistance did not change significantly. Moreover, a comparison of treated and untreated wastewater samples demonstrated that certain resistance genes, e.g., carbapenemase OXA-48 and the count of mobile genetic elements, were also not significantly reduced [6,26,27].

WWTPs are not specifically designed to remove the antibiotics ARB and ARGs [13]. The main requirement imposed on WWTPs is to ensure the optimum values of organic matter, nitrogen, and phosphorus, since the discharge of wastewater containing high levels of these elements may contribute to oxygen depletion and an increase in the trophic state of the receiving waters. However, the penetration of microbial contaminants with the treated wastewater is not usually subject to regulations or monitoring. It should be mentioned that, despite the reduction in the total number of bacteria and ARB in the wastewater treatment process, large numbers of bacteria exhibiting multi-drug-resistance characterized by higher virulence could still penetrate into the environment with the wastewater [6,28].

Since the presence of antibiotic resistant bacteria and antibiotic resistance genes in the environment poses a potential hazard to health, the present study applied molecular analyses for an assessment of the role of wastewater treatment plants in the spread of ARGs. Quantitative testing of genes encoding the resistance to beta-lactam antibiotics and tetracyclines (antibiotic classes most commonly used in medical treatment) enabled a performance assessment of a range of wastewater treatment plants that apply various modifications of the activated sludge-based treatment process. Genomic DNA samples originating from the population cultured in the study were used. A significant limitation of this method is the small amount of growth-capable environmental bacteria possible to obtain, although the aim of the study was to analyse antibiotic resistance in live bacterial cells with ARGs. The presence of live bacterial cells that are capable of multiplying constitutes a considerably greater potential threat of transmission of these genes from WWTPs to the environment. The study results may be of significance in identifying the degree of risks to the public health of humans and animals exploiting water bodies that receive treated wastewater.

2. Materials and Methods

2.1. Study Sites and Sampling

For the purposes of the study, untreated and treated wastewater samples were collected from thirteen wastewater treatment plants of various capacity, hydraulic retention time (HRT), and characteristics of the untreated wastewater, located in the Warmińsko-Mazurskie District in Poland. All of the samples were collected after the secondary treatment process. No additional wastewater disinfection was performed in the analysed WWTPs. Wastewater treatment plants were divided into four categories according to the applied modification of the wastewater treatment system: A—WWTPs (No. I, II) with an anaerobic-anoxic-aerobic (A_2O) bio-reactor system; B—WWTPs (No. III, IV, V, VI, VII) with a mechanical and biological system; C—WWTPs (No. VIII, IX, X) with SBRs (Sequencing Batch Reactors); and, D—WWTPs (No. XI, XII, XIII) with an increased nutrient removal mechanical and biological system (see Supplementary Material, Table S1). Wastewater samples were collected in the winter (February) to sterile bottles, transported to the laboratory at a temperature of 4 °C, and subjected to analysis on the day of collection [29]. The sample collection period was selected to correspond with an increased morbidity incidence and, thus, with higher antibiotic consumption.

2.2. Physicochemical Parameters and Number of Antibiotic-Resistant Bacteria

Physicochemical parameters of the wastewater samples under study, including five-day Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), and Total Suspended Solids (TSS), were assessed simultaneously with a microbiological analysis. The total number of bacteria as well as bacteria resistant to β-lactams (amoxicillin, cefotaxime) and tetracyclines (oxytetracycline, doxycycline) and the total number of *Escherichia coli* and *Escherichia coli* resistant to the same drugs were determined according to procedures that were described by Osińska et al. [29].

2.3. Bacterial Inoculation and DNA Extraction

To obtain 20–80 colony forming units (CFU) per plate, untreated (UWW) and treated wastewater (TWW) samples were decimally diluted with saline water and passed through a cellulose filter (pore diameter of 0.45 µM, Millipore, Merck KGaA, Darmstadt, Germany). Greater accuracy was achieved by plating in triplicates. The total number of bacteria and bacteria resistant to β-lactams (amoxicillin, cefotaxime) and tetracyclines (oxytetracycline, doxycycline), as well as the total number of *Escherichia coli* and *Escherichia coli* resistant to the same drugs were determined on plates with TSA medium (Oxoid, Thermo Fisher Scientific, Carlsbad, USA) and the mFc Agar medium (Merck, Merck KGaA, Darmstadt, Germany) with/without antibiotic supplementation, respectively. The plates were incubated at 30 °C for 48 h. *E. coli* were cultured at 44.5 ± 0.2 °C for 24 h. The filters were then transferred to sterile screw cap tubes (50 mL), and 30 mL of 1 × PBS was added to the tubes. They were shaken (200 rpm/min, three hours) at room temperature. The entire precipitate was then transferred to 2.0 mL Eppendorf tubes and centrifuged (9000 rpm/min, 15 min). DNA extraction from a bacterial pellet was then performed using isolation kits (Genomic Mini A&A Biotechnology kit, Gdynia, Poland), according to the manufacturer's instructions. The concentration and quality of the extracted DNA was determined by microspectrophotometry (NanoDrop® ND-1000, Nano Drop Technologies, Willmington, DE, USA). The DNA was stored at −20 °C for further analysis.

2.4. Quantification Analysis of ARGs

The obtained DNA samples originating from antibiotic resistant bacteria were used for ARG analysis with a conventional polymerase chain reaction (PCR) and a real-time polymerase chain reaction (qPCR) using specific primers. During preliminary testing, the occurrence of seven genes responsible for the resistance to tetracyclines (*tet*A, *tet*C, *tet*L, *tet*M, *tet*O, *tet*A(P), *tet*X) and ten genes responsible for the resistance to beta-lactams (bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M}, $bla_{CTX-M-1}$, bla_{CTX-2}, $bla_{CTX-M-9}$, bla_{VEB}, bla_{CMY}, bla_{AMP-C}) were investigated using the PCR reaction. These genes were

selected based on previous studies of the authors [11,28,30]. For ARG quantitative analysis with qPCR, genes (bla_{TEM}, bla_{SHV}, bla_{OXA}, *tet*A, *tet*M) were selected based on their occurrence in environmental samples in the preliminary testing. Moreover, the presence of gene *uid*A in *E. coli* was also used as a marker of the taxonomic genotype. Antibiotic resistance genes were used to create standard curves derived from wastewater strains. The standard curves were generated by cloning the amplicon from the positive control into vector pCR2.1-TOPO (Invitrogen, Massachusetts, USA). All qPCR reactions were carried out using a Roche Light Cycler 480 device (Roche Applied Science, Denver, CO, USA). The reactions were carried out in triplicate to ensure repeatability, using both the negative and positive control.

2.4.1. Preparation of Standard Curves

Prior to the preparation of serial dilutions to create the standard curve, it was necessary to calculate the number of copies of the gene under study. For this purpose, the following equation was applied:

$$\text{number of copies } = \frac{(\text{amount of DNA [ng]}) \times 6.022 \times 10^{23}}{\text{average size of genome[bp]} \times 10^{9} \times 650} \quad (1)$$

where: 650 g/mole means average molecular weight per base pair, 6.222×10^{23}/mole is Avogadro's number, including molecules, and 10^9 ng/g is the conversion factor [29]. Standard curves for qPCR were obtained within the range from 10^8 to 10^2 gene copies/µL. The negative control sample was distilled-deionised water (ddH_2O) added to the qPCR reaction mixture as a substitute for matrix DNA.

2.4.2. qPCR Reaction Conditions

All of the analysed genes were quantitatively determined with the qPCR method using SYBR Green (Roche Applied Science, Denver, CO, USA). Each sample was analysed in triplicate. After each qPCR assay, a melt curve was constructed by increasing the temperature from 65 to 95 °C to verify the specificity. The point of intersection of the fluorescence signal with the threshold line determined the cycle threshold (Ct).

All of the primers had been previously validated (for primer sequences, amplicon sizes, annealing temperatures, references for each sequence, and additional details regarding qPCR conditions see Supplementary Material, Table S2.

2.5. Data Analyses

Statistical analyses were carried out using the STATISTICA 10 software package (StatSoft Inc., 1984–2011). The p value of <0.05 was adopted as indicating significance. The microorganism count, gene concentration, and Spearman's rank correlation were analysed to identify correlations between the physicochemical parameters and HRT values for the treatment plants. Moreover, an ANOVA test was performed in order to identify the differences between the gene concentration in the DNA of populations originating from both treated and untreated wastewater from the analysed WWTPs.

3. Results

3.1. Concentration of Physicochemical Parameters and Number of Antibiotic-Resistant Bacteria

The physicochemical parameter values of the analysed wastewater samples and the total number of antibiotic-resistant bacteria and antibiotic resistant *E. coli* were published by Osińska et al. [29]. The average BOD values in the untreated wastewater samples ranged from 401 to 606 mg/L and following the wastewater treatment process they were reduced by 97–99.8%. Meanwhile, the observed COD values ranged from 1.199 to 1.540 mg/L in the UWW samples and, following the treatment process, their levels were reduced by 92–98%. The average total number of antibiotic-resistant bacteria in UWW ranged from 7.04×10^5 to 1.6×10^7 CFU/mL and from 3.32×10^4 to 2.75×10^6 CFU/mL for bacteria

resistant to beta-lactams and bacteria resistant to tetracyclines, respectively. These values were reduced by 68–99.9%, although the average count of antibiotic-resistant *E. coli* in untreated wastewater ranged from 3.67×10^2 to 4.45×10^5 and, following the treatment, it was reduced by 0–99.9%. Despite the high reduction in the antibiotic-resistant bacteria count, the percentage of these bacteria in the total number of microorganisms increased following the treatment process. The COD and BOD values were directly positively correlated with the number of analysed microorganisms ($p < 0.05$) (Table 1).

Table 1. Correlations between gene concentrations, total bacteria counts, WWTP parameters, and basic physico-chemical parameters.

	Spearman's Rank Coefficient												
	TSA/ 1 mL	**TSA-AMO/ 1 mL**	**TSA-CTX/ 1 mL**	**TSA-OX/ 1 mL**	**TSA-DOX/ 1 mL**	***tet*A**	***tet*M**	***bla*$_{TEM}$**	***bla*$_{OXA}$**	***bla*$_{SHV}$**	***uid*A**	**HRT**	**TSS**	**BOD**
TSA-AMO/1 mL	**0.95**													
TSA-CTX/1 mL	**0.94**	**0.94**												
TSA-OX/1 mL	**0.90**	**0.92**	**0.89**											
TSA-DOX/1 mL	**0.85**	**0.92**	**0.90**	**0.87**										
*tet*A	0.22	0.30	0.28	0.26	0.38									
*tet*M	0.20	0.21	0.28	0.20	0.29	0.13								
bla$_{TEM}$	**0.42**	0.34	0.36	0.27	0.30	**0.47**	0.03							
bla$_{OXA}$	**0.17**	0.16	0.20	0.19	0.21	0.02	**0.44**	0.15						
bla$_{SHV}$	**0.41**	0.37	**0.42**	0.32	**0.46**	**0.47**	**0.33**	**0.42**	**0.42**					
*uid*A	−0.01	0.00	0.03	−0.04	0.03	**0.39**	0.11	**0.68**	0.22	0.26				
HRT	−0.05	0.00	−0.02	−0.11	0.01	**0.41**	0.10	0.01	−0.28	0.31	**0.39**			
TSS	0.03	−0.04	−0.09	0.06	−0.04	−0.37	−0.15	0.29	0.08	0.08	**−0.40**	**−0.49**		
BOD	**−0.68**	**−0.72**	**−0.71**	**−0.62**	**−0.66**	−0.01	−0.27	−0.24	−0.18	−0.18	0.00	0.04	0.00	
COD	−0.30	−0.35	−0.34	**−0.43**	−0.38	**−0.45**	0.11	−0.18	−0.08	−0.15	0.30	0.19	−0.14	0.12

TSA—total bacteria counts, TSA+AMO—amoxicillin-resistant bacteria counts, TSA+CTX—cefotaxime-resistant bacteria counts, TSA+OX—oxytetracycline-resistant bacteria counts, TSA+DOX—doxycycline—resistant bacteria counts, HRT— Hydraulic Retention Time, TSS—Total Suspended Solids, BOD—Biochemical Oxygen Demand, COD—Chemical Oxygen Demand.

3.2. Quantitative Analysis of Antibiotic Resistance Genes and Taxonomic Genes

3.2.1. Quantitative Pccurrence of Antibiotic Resistance Genes among the Total Number of Antibiotic-Resistant Bacteria

Among the analysed DNA samples of bacterial populations from the TSA universal culture medium, the presence of all the tested genes responsible for the resistance to beta-lactam antibiotics and tetracyclines was detected, with the exception of four wastewater treatment plants (No. VII, VIII, XI, X), in which the *bla*$_{OXA}$ gene was not detected (Figure 1).

The genes occurring at the highest average concentrations were *tet*A (from 8.72×10^{-4} to 1.07 gene copies/gene copies 16S *r*RNA) as well as *bla*$_{OXA}$ (from 8.54×10^{-3} to 4.53×10^{-1} gene copies/16S *r*RNA gene copies) and *bla*$_{TEM}$ (from 8.33×10^{-4} to 1.49×10^{-1} gene copies/16S *r*RNA gene copies). The other genes responsible for antibiotic resistance occurred at a similar level, at concentrations of the order of 1×10^{-3}–1×10^{-2} gene copies/16S *r*RNA gene copies. An analysis of the correlations between concentrations of particular genes originating from the total bacterial population revealed a statistically significant correlation between the *bla*$_{SHV}$ gene and all antibiotic resistance genes. Moreover, there was also a positive correlation between *bla*$_{OXA}$ and *tet*M, and between *bla*$_{TEM}$ and *tet*A genes (Table 1). An analysis of the correlation between the concentration of particular genes and the count of microorganisms demonstrated a statistically significant correlation between the concentration of the *bla*$_{SHV}$ gene in the DNA of the total bacterial population and the microorganism count on TSA media, both with and without the supplementation with the tested antibiotics. However, no significant correlation was found between physicochemical parameters and the concentration of genes, or between HRT values of the analysed treatment plants and the concentration of genes in wastewater.

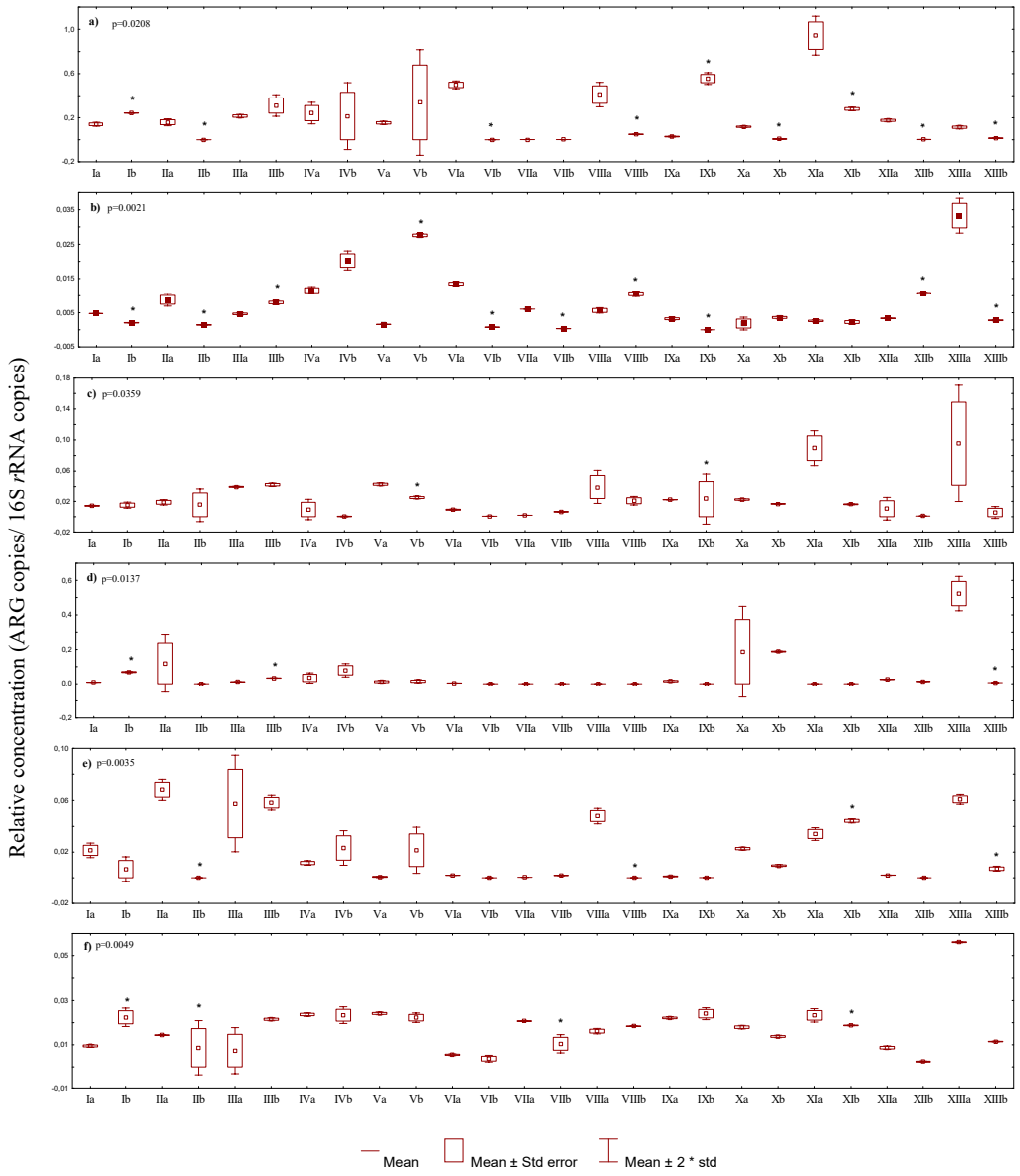

Figure 1. Relative antibiotic resistance gene (ARG) concentration in total bacteria population: (**a**) *tet*A (**b**) *tet*M (**c**) bla_{TEM} (**d**) bla_{OXA} (**e**) bla_{SHV} (**f**) *uid*A in collected wastewater samples. An asterisk (*) denotes a statistically significant difference (ANOVA; $p < 0.05$) between wastewater treatment plant (WWTP) untreated and treated samples. I-XIII–denote numbers of WWTPs. Group A (I, II), group B (III–VII), group C (VIII–X), group D (XI–XIII); a—untreated wastewater, b—treated wastewater.

Following the wastewater treatment process, the average concentration of genes that are responsible for the resistance to beta-lactams and tetracyclines decreased at levels ranging from 0 to 100% (Table 2).

A reduction in the concentration of all antibiotic resistance genes under study was observed in the DNA of the total bacterial population from WWTP No. VI, group B (mechanical and biological treatment plants), and WWTP No. XIII, group D (increased nutrient removal mechanical and biological treatment plants). Moreover, in the DNA of the total bacterial population from other group D WWTPs, an increase was observed in the concentration of one gene in treated wastewater, i.e., *tet*M in WWTP No. XII and bla_{SHV} in WWTP No. XI. Only in the DNA of the bacterial population from WWTP no. III, group B, was an increase noted in the concentrations of all the tested genes following the wastewater treatment process. However, in the DNA of the total bacterial population from WWTPS no. IV and V

(group B), an increase was observed in the concentrations of four analysed genes (*tet*A, *tet*M, bla_{OXA}, and bla_{SHV}). Of all the drug resistance genes under study, the concentrations of bla_{SHV} and bla_{OXA} were reduced with the highest effectiveness, reaching as much as 100%. The genes that are responsible for the resistance to tetracyclines proved to be the most problematic group, since for both genes, *tet*A and *tet*M, an increase in their concentration in the TWW was observed in the DNA of the total bacterial population from six WWTPs.

The genes occurring at the highest average concentrations were *tet*A (from 3.57×10^{-2} to 1.94 gene copies/16S *r*RNA gene copies) and bla_{OXA} (from 8.21×10^{-3} to 6.63×10^{-1} gene copies/16S *r*RNA gene copies). The gene *tet*M was present at lower concentrations (from 9.18×10^{-8} to 4.29×10^{-1} gene copies/16S *r*RNA gene copies). For other genes encoding the resistance to beta-lactams, the average concentrations ranged from 1×10^{-3} to 1×10^{-2} and from 1×10^{-5} to 1×10^{-4} gene copies/16S *r*RNA gene copies, for the genes bla_{TEM} and bla_{SHV}, respectively. An analysis of the correlations between the concentrations of genes in the *E. coli* population DNA demonstrated a positive correlation between gene *tet*M and genes *tet*A, bla_{OXA}, and bla_{SHV}, and between gene bla_{TEM} and *tet*A (Table 3).

Table 2. Gene concentration reduction in total bacteria population.

Sewage Treatment Technology Used	Treatment Plant	*tet*A	*tet*M	bla_{TEM}	bla_{OXA}	bla_{SHV}	*uid*A
A. WWTPs with A_2O system	I	*	56.66	*	*	36.97	*
	II	99.88	83.58	*	100	100	*
B. WWTPs with mechanical-biological system	III	*	*	*	*	*	*
	IV	*	*	96.73	*	*	1.21
	V	*	*	42.45	*	*	7.97
	VI	99.87	94.27	93.90	100	97.05	31.93
	VII	*	93.87	*	**	*	49.51
C. WWTPs with Sequencing Batch Reactors (SBR)	VIII	87.89	*	46.94	**	100	*
	IX	*	99.98	*	100	90.66	*
	X	94.46	*	25.24	**	58.49	23.50
D. WWTPs with mechanical-biological system with elevated removal of nutrients	XI	70.34	13.17	81.96	**	*	19.44
	XII	98.68	*	95.82	47.98	100	72.85
	XIII	87.16	91.55	88.59	98.78	88.34	79.56

*—increase in gene concentration, **—no gene presence, WWTP—wastewater treatment plant.

3.2.2. Quantitative Occurrence of Antibiotic Resistance Genes among Antibiotic-Resistant *E. coli*

In the DNA of the bacterial population from *E. coli* selective medium mFc, *tet*M, *tet*A, and bla_{OXA} genes were not detected in untreated wastewater from all treatment plants (Figure 2).

Following the wastewater treatment process, the average concentration of genes that are responsible for the resistance to beta-lactams and tetracyclines decreased at a level ranging from 0 to 100% (Table 4).

A complete reduction in genes was observed for the *tet*A gene in the DNA of *E. coli* population from four wastewater treatment plants (No. VII, VIII, X, XI) and for the bla_{OXA} gene for the DNA of *E. coli* population isolated from WWTP No. III. A reduction in all genes under study was observed only in the DNA of *E. coli* populations from WWTPs No. VIII and IX, group C, ranging from 21% to 100%. An increase in the concentration of all genes under study following the wastewater treatment process was observed in the DNA of *E. coli* population from WWTP No. IV, group B. In addition, an increase in the concentrations of four genes under study (*tet*A, *tet*M, bla_{OXA}, bla_{TEM}) was also observed in the DNA of *E. coli* population in WWTP No. V, group B, and gene bla_{SHV} was reduced only by 8%. Wastewater treatment plants reduced the concentration of the bla_{OXA} gene most efficiently. However, the bla_{TEM} gene, whose concentration increased following the treatment process, was noted in the

DNA of the population of *E. coli* from as many as eight WWTPs and was the most difficult to remove from wastewater.

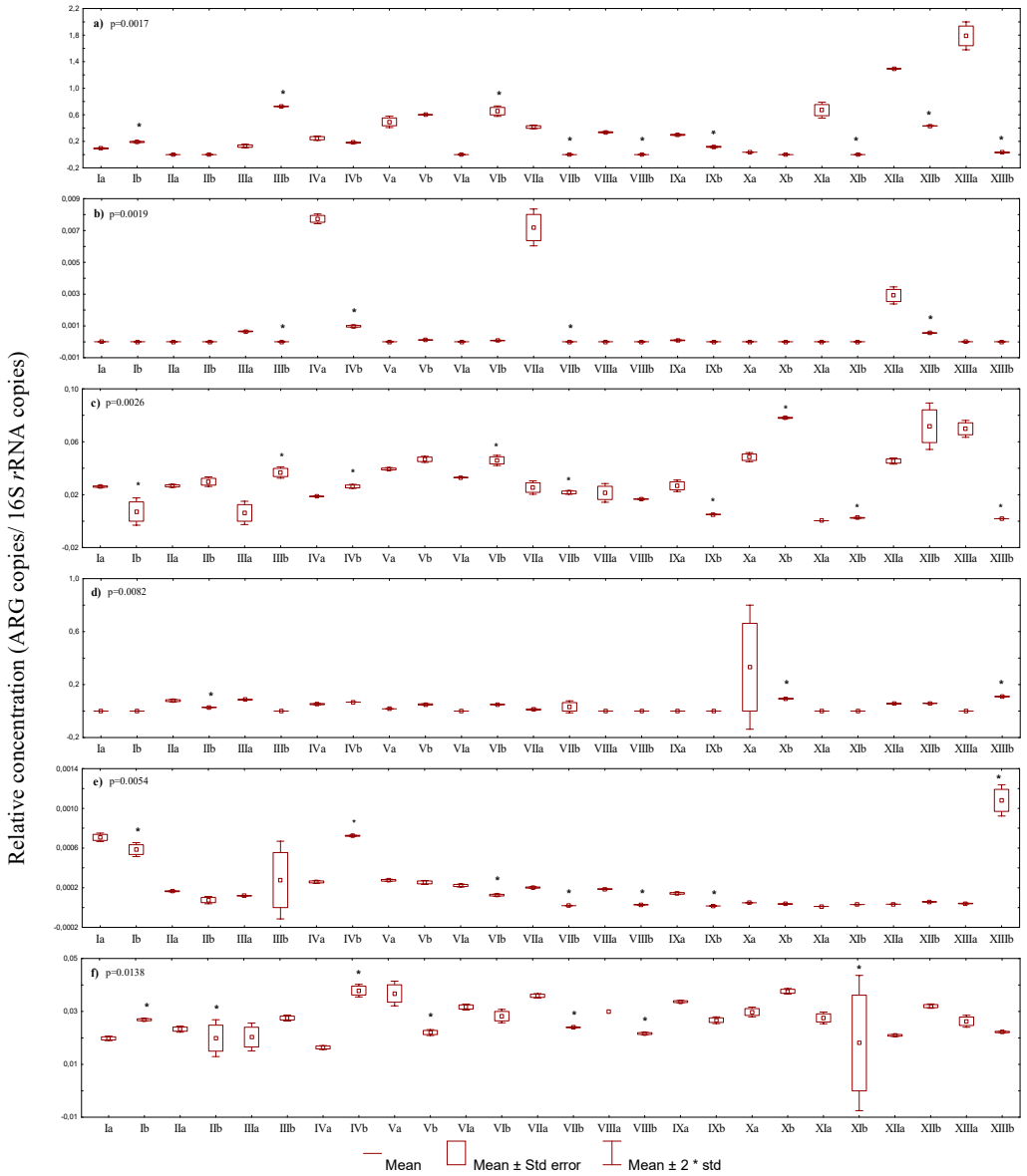

Figure 2. Relative ARGs concentration in *E. coli* population: (**a**) *tet*A (**b**) *tet*M (**c**) bla_{TEM} (**d**) bla_{OXA} (**e**) bla_{SHV} (**f**) *uid*A in collected wastewater samples. An asterisk (*) denotes a statistically significant difference (ANOVA; $p < 0.05$) between WWTP influent and effluent samples. I-XIII—denote number sof WWTPs. Group A (I, II), group B (III–VII), group C (VIII–X), group D (XI–XIII); a—untreated wastewater, b—treated wastewater.

Table 3. Correlations between gene concentrations, *E. coli* counts, WWTP parameters, and basic physico-chemical parameters.

	Spearman's Rank Coeffcient													
	mFc/ 1 mL	**mFc-AMO/ 1 mL**	**mFc-CTX/ 1 mL**	**mFc-OX/ 1 mL**	**mFc-DOX/ 1 mL**	***tet*A**	***tet*M**	***bla*$_{TEM}$**	***bla*$_{OXA}$**	***bla*$_{SHV}$**	***uid*A**	**HRT**	**TSS**	**BOD**
mFc-AMO/ 1 mL	**0.93**													
mFc-CTX/ 1 mL	**0.66**	**0.74**												
mFc-OX/ 1 mL	**0.81**	**0.83**	**0.59**											
mFc-DOX/ 1 mL	**0.68**	**0.69**	**0.65**	**0.73**										
*tet*A	0.24	0.13	0.13	0.14	0.07									
*tet*M	0.17	0.14	0.17	0.05	−0.02	**0.56**								
bla$_{TEM}$	0.16	0.07	−0.11	0.09	0.13	**0.30**	0.24							
bla$_{OXA}$	−0.09	−0.02	−0.12	0.02	−0.03	−0.12	**0.28**	0.22						
bla$_{SHV}$	0.10	0.13	0.15	0.01	0.00	0.05	**0.37**	−0.02	0.21					
*uid*A	−0.04	−0.12	−0.32	−0.15	−0.06	0.11	0.06	**0.32**	−0.04	0.02				
HRT	−0.05	−0.08	0.08	−0.11	−0.23	0.03	0.14	**−0.61**	−0.38	−0.10	−0.12			
TSS	−0.01	0.06	**0.46**	0.14	0.23	−0.03	−0.31	−0.05	−0.12	−0.19	−0.01	−0.07		
BOD	**−0.63**	**−0.63**	**−0.69**	**−0.54**	**−0.43**	−0.31	−0.35	−0.11	−0.13	−0.23	0.05	0.04	−0.15	
COD	−0.29	−0.38	−0.28	**−0.41**	−0.34	0.20	0.27	0.17	−0.21	−0.09	0.25	0.19	−0.28	0.12

mFc—total *E. coli* counts, mFc+AMO—amoxicillin-resistant *E. coli* counts, mFc+CTX—cefotaxime-resistant *E. coli* counts, mFc+OX—oxytetracycline-resistant *E. coli* counts, mFc+DOX—doxycycline-resistant *E. coli* counts, HRT—Hydraulic Retention Time, TSS—Total Suspended Solids, BOD—Biochemical Oxygen Demand, COD—Chemical Oxygen Demand.

Table 4. Gene concentration reduction in *E. coli* populations.

Sewage Treatment Technology Used	**Treatment Plant**	***tet*A**	***tet*M**	***bla*$_{TEM}$**	***bla*$_{OXA}$**	***bla*$_{SHV}$**	***uid*A**
A. WWTPs with A_2O system	I	*	93.66	44.30	**	17.50	*
	II	**	**	*	64.30	53.95	14.67
B. WWTPs with mechanical-biological system	III	*	99.66	*	100	*	*
	IV	*	*	*	*	*	*
	V	*	*	*	*	7.63	40.10
	VI	**	*	*	**	43.52	10.82
	VII	100	99.997	14.50	*	90.47	33.22
C. WWTPs with Sequencing Batch Reactors (SBR)	VIII	100	86.13	21.88	**	84.69	27.77
	IX	60.98	99.76	80.98	**	88.40	20.75
	X	100	68.71	*	85.93	26.06	*
D. WWTPs with mechanical-biological system with elevated removal of nutrients	XI	100	*	*	**	*	*
	XII	66.62	80.96	*	*	*	*
	XIII	98.21	19.55	97.30	**	*	15.52

*—increase in gene concentration, **—no gene presence, WWTP—wastewater treatment plant.

3.2.3. Quantitative Occurrence of *uid*A Genes

The average concentration of the taxonomic gene of *E. coli* ranged from 9.97×10^{-3} to 5.63×10^{-2} gene copies/16S *r*RNA gene copies for the DNA from the total population of bacteria isolated on TSA medium, and from 1.50×10^{-2} to 4.29×10^{-1} gene copies/16S *r*RNA gene copies for the DNA derived from the population of *E. coli* isolated on the mFc medium. The concentrations of genes *uid*A in the DNA of the total bacterial population were positively correlated with the concentration of genes *tet*A and *bla*$_{TEM}$, while concentrations of genes *uid*A in the DNA of *E. coli* population were only positively correlated with gene *bla*$_{TEM}$. The greatest reduction in the concentration of *E. coli*, based on the taxonomic gene, was observed in WWTP No. XII, group D (80%) in the DNA of the total bacterial population and in WWTP No. V, group B (40%) for the DNA of *E. coli* population. The relative concentration of gene *uid*A following the wastewater treatment process increased for the DNA

obtained from *E. coli* population in six wastewater treatment plants, while in the DNA from the total bacterial population, from five WWTPs.

4. Discussion

Wastewater treatment plants constitute a potential source of ARB and ARGs, which can be transmitted to the environment with discharged treated wastewater. For this reason, monitoring microbiological pollutant removal effectiveness during wastewater treatment is of particular significance. Results of studies on the number of antibiotic resistant bacteria on culture media were published by Osińska et al. [29]. Based on these results, the most frequently occurring bacteria were those that were resistant to amoxicillin and oxytetracycline, i.e., older generation antibiotics. Even though wastewater treatment plants usually ensured a rather high level of reduction in the number of antibiotic-resistant bacteria (at least 99.9%), treated wastewater from WWTPs that were discharged to the environment contained, on average, from 1.05×10^2 to 2.77×10^5 CFU/mL. It should be stressed that the percentage of antibiotic-resistant bacteria in the total number of microorganisms increased following the wastewater treatment process.

Genes that are responsible for resistance to beta-lactam antibiotics were reduced to a greater extent following the wastewater treatment process as compared to the number of genes responsible for resistance to tetracyclines. Czekalski et al. [6] also found that, despite a reduction by as much as 78% in the total number of bacteria following the wastewater treatment process, a reduction in ARGs concentration was not always observed. Moreover, the occurrence of multi-drug resistant bacteria was found. Rafrat et al. [31] demonstrated that the concentration of $bla_{\text{CTX-M}}$, bla_{TEM}, and *qnr*S genes relative to the number of copies of 16S *r*RNA was generally similar in the samples of wastewater both before and after the treatment process. The authors also demonstrated that in wastewater from the treatment plant receiving mixed municipal and hospital wastewater, the concentration of genes increased following the treatment process. In wastewater from a biological treatment plant using disinfection with UV radiation, there was also no reduction in the concentrations of bla_{TEM}, *qnr*A, and *sul*I genes in the samples of treated wastewater from that WWTP. Rodriguez-Mozaz et al. [23] noted that the relative concentrations of *erm*B and *tet*W genes decreased as a result of wastewater treatment, while the concentration of bla_{TEM}, *sul*1, and *qnr*S genes in wastewater increased. Those authors also demonstrated that the incomplete removal of antibiotics and ARGs in the wastewater treatment process significantly affected the water quality of the river receiving treated wastewater. This was because the concentration of antibiotics and ARG concentrations at a point beyond the wastewater inflow were higher than in the samples collected before the effluent discharge point. Other study results [24,25] also confirm the ineffective elimination of ARGs by conventional wastewater treatment plants. They also indicate that the bacteria released with wastewater from WWTPs may be capable of actively spreading the resistance genes among the local microorganisms, thus inhabiting ecosystems of surface water bodies that receive treated wastewater.

The present study demonstrated that for the genes responsible for resistance to tetracyclines in all tested WWTPs, both in the total bacterial population and in *E. coli* population, the *tet*A gene occurred at higher concentrations than the *tet*M gene. Zhang et al. [18]. in their study concerning the change in microorganism communities of the activated sludge and ARGs under selection pressure of antibiotics in SBR reactors, observed that the total number of ARGs in the activated sludge increased following the addition of tetracycline (TC). This indicates that bacteria with ARGs proliferate intensively under the pressure of antibiotic presence. Moreover, the study demonstrated that, with an increase in TC concentration, the concentration of genes that are mainly responsible for the efflux pump (*tet*A, *tet*B, *tet*C) increased, while the concentration of genes responsible for the abundance of ribosomal genes of protective proteins (*tet*M, *tet*O, *tet*S) was lower when compared with the number of the efflux pump genes and that this number changed slightly with an increase in TC concentration.

The present study also noted the highest concentrations of *tet*A and bla_{OXA} genes in the DNA of *E. coli* populations, while the occurrence of *tet*M and bla_{OXA} genes was not noted in all the studied

samples of the DNA of the *E. coli* populations from wastewater treatment plants. As a result of the intake of antibiotics, antibiotic resistance genes can be modified and acquired by the microbiota inhabiting the gastrointestinal tract, which may be released to the environment with faeces via drainage systems [23]. Osińska et al. [28] found that, following the wastewater treatment process, the frequency of the occurrence of antibiotic resistance genes increased among *E. coli* bacteria. The authors also noted an increase in the percentage of multi-drug resistant *E. coli* as compared to untreated wastewater. Hendricks and Pool [32] suggest that wastewater treatment plants are ineffective in eliminating bacteria, including faecal contamination indicator bacteria, even though studies with opposite results have been reported [30]. These great differences in the efficiency of processed wastewater treatment may result from the wastewater treatment plant capacity and/or the type of wastewater [33]. Hembach et al. [4] also found a slight reduction in the concentration of ARGs during standard wastewater treatment at seven wastewater treatment plants of different capacities.

The conducted study indicated concentrations of *uid*A gene that enable the determination of *E. coli* of an order up to 10^{-1} copies/16S *r*RNA gene copies. In most cases, no significant reductions in the number of copies of this gene following the wastewater treatment process were noted. Rafrat et al. [31] demonstrated that the number of copies of bacterial 16S *r*RNA was up to 5.84×10^8 copies/mL of sample, and there were slight differences ($p > 0.05$) between the number of gene copies in untreated and treated wastewater. The results of the studies of those authors suggest that the applied wastewater treatment processes were not effective in reducing the bacterial load, which is also true for wastewater treatment plants applying UV disinfection following the conventional treatment process. Even though a study by Hendricks and Pool [32] demonstrated that wastewater treatment plants may decrease the load of nutrients and faecal coliforms from treated wastewater, Rafrat et al. [31] indicated that the total abundance of bacteria (estimated based on the number of copies of the bacterial gene 16S rRNA) was not reduced following the treatment process, irrespective of the process applied. Therefore, biological wastewater treatment plants may provide an appropriate environment for the spread of ARGs, as both the conditions prevailing during wastewater treatment, and the great diversity of microorganisms facilitate the transmission of genes through, *inter alia*, horizontal gene transfer (HGT) [6].

For most WWTPs receiving only municipal wastewater, the bla_{OXA} gene was not detected in the DNA obtained from the total population of bacteria isolated on TSA medium or in the DNA of the *E. coli* population. In the DNA of populations that were isolated from wastewater in other treatment plants, the concentration of this gene was dominant. Another quantitatively dominant gene responsible for resistance to beta-lactams was the bla_{TEM} gene. It is believed that the group of TEM-type beta-lactamases may be of paramount significance in the development of resistance to beta-lactam antibiotics due to its great diversity (over 220 types of genes belong to this group) [34]. The results that were obtained by Ojer-Usoz et al. [35] also indicated the dominant occurrence of bla_{TEM} gene in relation to the bla_{SHV} and bla_{OXA} genes in the DNA of isolates originating from wastewater treatment plants. However, this study found that WWTPs that received only municipal wastewater proved to exhibit a much better performance in reducing the analysed genes responsible for antibiotic resistance than wastewater treatment plants also receiving wastewater from the food industry and hospitals.

Following the wastewater treatment process, an increase or a slight reduction in the concentration of studied ARGs was observed in the DNA of the total bacterial population and in the DNA of the *E. coli* population, mainly in WWTPs with a mechanical and biological system (group B). Moreover, an increase in the concentration of all the studied genes was observed in the DNA of the total bacterial population from WWTP No. II and in the DNA of the *E. coli* population isolated from WWTP No. IV. An increase in the ARG concentration following the wastewater treatment process was also found in the DNA samples from both studied populations isolated from WWTP No. V with a mechanical and biological system. Moreover, an over 90% decrease in all ARGs in the DNA of the total bacterial population was noted in WWTP No. VI, which used a mechanical and biological wastewater treatment system. At the same time, an increase in the number of *tet*M and bla_{TEM} gene copies and the presence of *tet*A gene was observed in the DNA of the *E. coli* populations that

were isolated from the same plant. Interestingly enough, the latter gene was not detected in the untreated wastewater received by the plant. The highest reduction in the studied ARGs was recorded for the WWTP No. XII (group D) with an increased nutrient removal mechanical and biological system in the DNA samples isolated from the total bacterial populations and for the WWTPs No. VIII and IX with sequencing batch reactors (group C) in the DNA samples that were obtained from the *E. coli* populations. Rafrat et al. [31] also found differences in the concentrations of genes in treated wastewater from various WWTPs, including an increase in the concentration of genes in wastewater from a treatment plant which receives municipal and hospital wastewater. The differences in ARG concentrations between wastewater samples from various treatment plants may be due to the differences in microbiological composition of wastewater incoming to particular treatment plants and the diversity of antibiotics (concentration and type) present in wastewater [17]. Guo et al. [36] found that ARGs from chromosomal mutations, under the great selective pressure of antibiotics, decreased along with the degradation of total antibiotics in final effluents, instead of proliferating through biological treatment stages. Youan et al. [35] demonstrated that the ARB count and the ARG number were statistically significantly positively correlated with the COD and the contaminant load, and negatively correlated with the oxygen content and the wastewater temperature. Moreover, Jiao et al. [37] observed that the absolute concentration of ARGs decreased by one order of magnitude in the DNA of samples that were collected in winter as compared with the DNA samples collected in summer, which could be explained by the fact that low temperatures may cause microbial enzyme deactivation, thus having a direct effect on ARG removal effectiveness. These correlations were not confirmed by Wen et al. [25], who suggest that varied temperatures throughout different seasons does not have a significant effect on the ARG concentration. These differences may be explained by regional diversification of antibiotic doses or by other factors that are connected with seasonal temperature fluctuations. A factor with a significant effect on the ARG concentrations may also be the composition of the wastewater reaching WWTPs. Numerous studies have documented high concentrations of ARGs in wastewater produced by hospitals [6,23,30] or some industries, e.g., the food industry [11,20,38]. Moreover, dissemination of ARGs may also be affected by wastewater contamination with heavy metals, whose residues may pose co-selection pressure on ARG distribution [24]. For this reason, ARGs are not effectively eliminated in the wastewater treatment process and their concentration may even increase [39]. Wastewater discharge to the environment or their re-utilisation may lead to the dissemination of ARB and ARGs in the environment, threatening the ecological safety of aquatic ecosystems, in particular [27,40]. Moreover, the continued ARB and ARG discharge to the environment may contribute to an increase in infections with resistant pathogens and may enlarge the ARG pool among the environmental bacteria [6]. At the same time, Lorenzo et al. [41] demonstrated that, despite an effective reduction in absolute ARB and ARG concentrations by wastewater treatment plants, they might still remain hot-spots of antibiotic resistance expansion among wastewater bacteria sensitive to these drugs and considerably increase the antibiotic resistance levels in freshwater ecosystems that receive such wastewater. The results of the present study of the role of wastewater treatment plants in antibiotic resistance have demonstrated that the ARG concentrations in wastewater discharged from WWTPs may fluctuate significantly and the factors determining ARG elimination effectiveness in the wastewater treatment process should be monitored.

5. Conclusions

The results of the present study indicate that WWTPs play a significant role in the acquisition and spread of antibiotic resistance genes among bacterial populations. The presence of ARGs in live bacterial cells that are capable of multiplying constitutes a considerably greater potential threat of transmission of these genes from WWTPs to the environment. Therefore, the present study may be helpful in identifying the risks to human public health and the well-being of animals exploiting water bodies that receive treated wastewater. The analysed genes of resistance to beta-lactam antibiotics (bla_{OXA}, bla_{TEM}, bla_{SHV}) and to tetracyclines (*tet*A, *tet*M) were detected in the DNA of

bacterial populations isolated from wastewater from most wastewater treatment plants under study. In the DNA of *E. coli* population, the occurrence of *tet*M and bla_{OXA} genes was not detected in any of the tested WWTPs. It should be noted that not all gene concentrations were reduced following the treatment processes. For certain genes (bla_{TEM}, *tet*A), their average concentrations increased by as much as one order of magnitude following the treatment process. The results of this study indicate the presence of significant concentrations of genes that are responsible for antibiotic resistance, even in the treated wastewater from WWTPs. Generally, in wastewater treatment plants with a mechanical-biological system, an increased number of ARGs copies after treatment processes were observed. This indicates the need for continuous monitoring of wastewater treatment processes, as the discharge of wastewater to aquatic environments following treatment processes may pose a serious hazard to environmental safety.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/9/3/387/s1, Table S1: Technical description of wastewater treatment plants (WWTPs) and physicochemical parameters of wastewater, Table S2: Quantitative PCR (qPCR) primers and conditions used in this study, Table S3: Correlations between concentration of genes, total number of bacteria, WWTP parameters and basic physico-chemical parameters, Table S4: Correlations between concentration of genes, number of *E. coli*, WWTP parameters and basic physico-chemical parameters.

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