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Evaluation of the Effectiveness of the Activated Sludge Process in the Elimination Both ATB-Resistant and ATB-Susceptible *E. coli* Strains

Iwona Beata Paśmionka [1,*], Karol Bulski [1], Piotr Herbut [2,3], Elżbieta Boligłowa [1], Frederico Márcio C. Vieira [3], Gabriela Bonassa [4], Marina Celant De Prá [4] and Marcelo Bortoli [4]

[1] Department of Microbiology and Biomonitoring, Faculty of Agriculture and Economics, University of Ariculture in Krakow, 31-120 Kraków, Poland; Karol.Bulski@urk.edu.pl (K.B.); elzbieta.boliglowa@urk.edu.pl (E.B.)
[2] Department of Rural Building, Faculty of Environmental Engineering and Land Surveying, University of Agriculture in Krakow, 31-120 Kraków, Poland; p.herbut@ur.krakow.pl
[3] Grupo de Estudos em Biometeorologia—GEBIOMET (Biometeorology Study Group), Universidade Tecnológica Federal do Paraná (UTFPR), Estrada para Boa Esperança, km 04, Comunidade São Cristóvão, Dois Vizinhos 85660-000, Brazil; fredericovieira@utfpr.edu.br
[4] Grupo de Pesquisa em Gerenciamento e Tratamento de Resíduos—GETRAT, Universidade Tecnológica Federal do Paraná (UTFPR), Estrada para Boa Esperança, km 04, Comunidade São Cristóvão, Dois Vizinhos 85660-000, Brazil; gabrielabonassa@gmail.com (G.B.); marinapra@utfpr.edu.br (M.C.D.P.); marcelobortoli@utfpr.edu.br (M.B.)
* Correspondence: iwona.pasmionka@urk.edu.pl; Tel.: +48-126624400

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Abstract: Water reuse is now becoming a global necessity. However, one of the drawbacks in releasing wastewater into the environment is some persistent pollutants that are not completely removed in wastewater treatment plant. Residual bacteria and antibiotics in the inflowing wastewater can contribute to the antibiotic resistance spread in the aquatic environment. This study determined the effectiveness of activated sludge process for fecal coliform bacteria elimination, and also the *Escherichia coli* resistance to antimicrobial agents as erythromycin, azithromycin, clarithromycin, ofloxacin, ciprofloxacin, trimethoprim, and metronidazole in treated wastewater. The research was carried out using the membrane filtration technique, and the susceptibility of isolates to antimicrobial agents was tested by the disc diffusion method. The concentrations of fecal coliform bacteria and *Escherichia coli* differed significantly depending on the seasonal period in which it was carried out. Despite up to 99% reduction in the number of sanitary indicators in biologically treated wastewater, 89% of *E. coli* isolates resistant to the tested antibiotics was found, while 100% of the isolates were susceptible to metronidazole. Most of the isolates showed resistance to trimethoprim, and the fewest isolates were resistant to ofloxacin, indicating that some strains may react differently to antibiotics.

Keywords: wastewater; wastewater treatment plant; activated sludge; fecal indicators; *Escherichia coli*; removal performance; antibiotics; antibiotic resistance

1. Introduction

The constantly deepening world water deficit makes it necessary to ensure the appropriate quality of available water resources. The unquestionable benefits of wastewater treatment plants are followed by certain difficulties. The most important of which are the problems of wastewater sludge management and discharging to water bodies. Microbiological contamination is one of the basic threats to surface waters which are receivers of wastewater and potential sources of drinking water. Apart from physical and chemical pollutants, wastewater also contains numerous pathogenic and opportunistic microorganisms, mainly of intestinal origin. Raw wastewater is the largest reservoir of human intestinal bacteria. In addition, treated wastewater, even with the use of highly effective methods, may pose a serious microbiological threat to the receiving waters in terms of bacteriology.

Therefore, one of the most important current technological challenges is effective wastewater treatment, which will ensure not only the appropriate physicochemical quality of the effluents, but also the microbiological condition that is safe in terms of sanitation.

Among the potential environmental threats related to the spread of waterborne microorganisms is the increasing phenomenon of antibiotic resistance. Wastewater is an important reservoir of antibiotic resistance genes [1,2], which also contain antibiotics and their metabolites, contributing to the selection pressure. This phenomenon leads to the elimination of susceptible bacterial strains and the selection of resistant ones that spread in the ecological niche that has emerged. It has been shown that in wastewater treatment processes based on the activated sludge method, a positive selection of bacteria resistant to antibiotics takes place [3–6], e.g., selection of *E. coli* isolates resistant to penicillins, fluoroquinolones, and trimethoprim-sulfamethoxazole [7,8]. Moreover, the transformation of plasmid DNA obtained from selected *E. coli* isolates from raw and purified wastewater indicates a possible transfer of resistance genes to β-lactam antibiotics and tetracycline [9]. More and more attention is devoted to understanding trends in acquired antibiotic resistance among bacteria living in the natural environment. There is a high probability that the traits of resistance can be transferred mainly as a result of horizontal gene transfer between bacterial populations from natural environments and the populations brought into the environment that are part of the saprophytic and pathogenic human microflora [10]. In this process, commensal bacteria are considered to be potential resistance gene transfer vectors.

Antimicrobial resistance poses a serious threat to the global public health system. Environmental contamination with pharmaceuticals increases as a result of improper or excessive use of antimicrobial substances and problems with their disposal from wastewater. As a consequence, more and more waters are contaminated with drugs, including antibiotics [11], leading to an increase in the number of antibiotic resistant bacterial strains.

In recent years, the role of wastewater treatment plants in transferring bacterial resistance to antibiotics has been widely discussed [12–14]. Antibiotics and antibiotic-resistant bacteria reach treatment plants with various municipal, hospital, and industrial wastewater. The high concentration of bacteria in the activated sludge promotes the processes of horizontal gene transfer. Due to the presence of pharmaceuticals, heavy metals, and other inhibitory compounds in the activated sludge reactor, there may be a selection pressure that favors resistance to antibiotics [15–17]. Despite the research intensification, the issue of the potential spread of antibiotic resistance through wastewater treatment plants remains unresolved.

Due to the possible impact of antibiotic substances on living organisms, measures should be taken to monitor the presence of these compounds in various environments. An important issue is also a thorough understanding of the ecology of bacteria resistant to antimicrobial agents. Microbiological indicators such as enteric sticks are commonly used in the sanitary assessment of the environment, and in particular, the number of *Escherichia coli* is monitored.

It is extremely important to analyze the presence of antibiotic resistance directly in the wastewater treatment plant, as well as in the treated wastewater, which is then discharged into surface waters. This allows the actual impact of the treatment plant on the spread of antibiotic resistance in the environment to be determined. The spread of antibiotic resistance is calling into question the future of many treatments. The status of antibiotic-resistant bacteria and genes that determine antibiotic resistance in environments receiving treated wastewater remains unclear. Therefore, the study focuses on the microbiological hazards of outflow from wastewater treatment plants, including the occurrence and selection of antibiotic-resistant isolates in wastewater treatment processes and the potential transfer of resistance features to the water environment of receivers.

The aim of the research was to determine the effectiveness of biological wastewater treatment in the elimination of fecal coliform bacteria (thermotolerant), including *Escherichia coli*, and to compare the susceptibility to selected antibiotics of *Escherichia coli* isolates present in treated wastewater at the WWTP in Oświęcim in Poland.

2. Materials and Methods

2.1. Treatment Plant Characteristics

The tests were conducted at the Municipal and Industrial Sewage Treatment Plant in Oświęcim (50°02′17.1″ N 19°19′13.8″ E). The treatment plant is located in south-eastern Lesser Poland and collects municipal wastewater from the town and commune of Oświęcim and industrial wastewater from the nearby Chemical Production Plants. The current amount of municipal wastewater is about 14,000 m^3 per day and includes:

1. Domestic wastewater and wastewater from small production enterprises—11,320 m^3 per day;
2. Infiltration waters—2300 m^3 per day;
3. Wastewater delivered from septic tanks—380 m^3 per day.

The amount of industrial wastewater from the Chemical Production Plants is approximately 26,400 m^3 per day.

The composition of wastewater flowing into the treatment plant during the research period is shown in Table 1.

Table 1. Wastewater composition.

Sampling Date	pH	BOD$_5$ (mg·dm^{-3})	COD (mg·dm^{-3})	Total Suspended Solids (mg·dm^{-3})	Total Nitrogen (mg·dm^{-3})	Total Phosphorus (mg·dm^{-3})
Spring	7.7	406.64	663	337	46.9	8.44
Summer	7.7	454.71	733	253	43.9	8.98
Autumn	7.6	232.14	956	174	52.5	8.84
Winter	7.8	199.66	883	388	50.1	6.18

Initial treatment of municipal and industrial wastewater takes place in separate technological lines consisting of a grate, sand trap, and preliminary settling tanks. Post-production wastewater is additionally subjected to the process of neutralization and coagulation. After mixing with municipal wastewater in a 2:1 ratio, it is sent for biological treatment.

Biological treatment is based on the technology of low-loaded activated sludge. The biological system consists of an anaerobic reactor, four aeration reactors, three secondary radial settling tanks, a blower station, and an activated sludge pumping station (Figure 1).

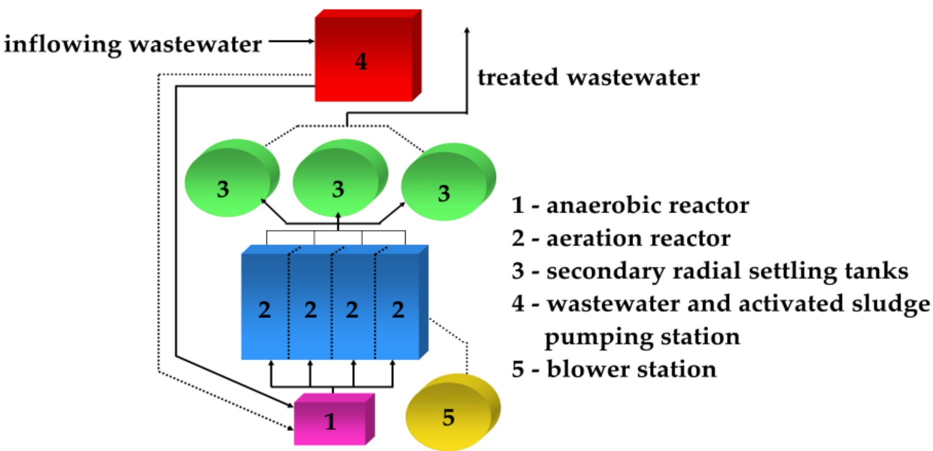

Figure 1. Diagram of the biological wastewater treatment system in the Municipal and Industrial Wastewater Treatment Plant in Oświęcim, Poland.

The treated wastewater together with the activated sludge flows by gravity to three secondary radial settling tanks. In the secondary settling tank, the activated sludge is separated from the wastewater. The settled activated sludge is collected into the central funnel and flows to the reactor, from which it is returned to the process as recirculated sludge or is pumped out as excess sludge. The treated wastewater is discharged to the Macocha stream, which is a tributary of the Vistula River.

2.2. Sampling and Bacteriological Contamination Analysis

The research was conducted in the period from March 2020 to February 2021. The samples consisted of inflowing and biologically treated wastewater. Five hundred cubic centimeters of wastewater were collected in the spring, summer, autumn, and winter periods, in triplicate. A total of 24 wastewater samples were collected (12 inflowing wastewater samples and 12 treated wastewater samples), two samples per month. The inflowing wastewater samples representing industrial and raw municipal wastewater (mixed 2:1) were pretreated by sand removal process. The treated wastewater samples were collected at the end of the wastewater discharge channel to the receiver. Chilled samples were taken to the laboratory for immediate analysis.

The presence of the tested bacteria in the samples was assessed using the membrane filtration method on Endo agar (Emapol, Gdańsk, Poland) in accordance with the adopted standards [18]. The samples were filtered through 0.47-mm cellulose acetate membrane filters with a nominal pore size of 0.45 μm (Sartorius, Göttingen, Germany) using a Sartorius filtration kit and a Rocker 300 vacuum pump. Depending on the wastewater concentration, prior to filtration, 10-fold serial dilutions up to 1:1,000,000 in physiological NaCl solution were made. A maximum of 100 cm^3 of the undiluted sample was filtered. Each volume was filtered in triplicate. The filters were transferred to 50-mm Petri dishes containing Endo agar and incubated at 44 °C. After 24 h of incubation, fecal coliforms were counted using the eCount Colony Counter (Heathrow Scientific) and the results were shown as CFU$\cdot$ 100 cm^{-3} (CFU-colony forming units).

2.3. Escherichia coli Isolation and Identification

Pure cultures of *E. coli* were isolated from samples of treated wastewater in which the presence of thermotolerant coliform bacteria was detected. For this purpose, inoculation on Eosin-Methylene-Blue agar (Biocorp, Issoire, Poland) was used. After 24 h of incubation at 44 °C, purple-colored colonies with a green metallic luster (similar to *Escherichia coli*) were transferred to TBX agar (BTL, Warszawa, Poland). Tryptone Bile X-glucuronide Agar is a chromogenic medium on which *E. coli* grows at 44 °C in the form of blue-green colonies. Systematic position of isolates was determined based on biochemical properties [19] using API20E tests (Bio-Merieux, Marcy l'Étoile, France).

2.4. Antibiotic Resistance Testing

In order to investigate the susceptibility of *E. coli* isolates to antibiotics, disc diffusion tests (BIO-RAD, Hercules, CA, USA) were performed on Mueller–Hinton II medium (BTL, Warszawa, Poland). Sensitivity assessment and interpretation of results were performed according to the guidelines of KORLD (National Reference Center for Susceptibility Testing) and CLSI (Clinical and Laboratory Standards Institute) [20]. Inhibition diameters of growth were measured after 18–20 h of incubation at 37 °C. The results were compared with the breakpoint values provided by EUCAST (European Committee on Antimicrobial Susceptibility Testing) [21].

The discs used contained the antimicrobials most often detected in WWTPs: erythromycin (ERY 15 μg), azithromycin (AZM 15 μg), clarithromycin (CLR 15 μg), ofloxacin (OFX 5 μg), ciprofloxacin (CIP 5 μg), ampicillin (AMP 10 μg)), trimethoprim (TMP 5 μg), and metronidazole (MTR 50 μg) [22]. *E. coli* ATCC 25922 strain was used for quality control.

2.5. Data Analysis

In order to determine the significance of differences between the amount of fecal coliforms and *E. coli* isolated from wastewater, a one-way ANOVA was used, and Tukey's test was used to verify the differences in the number of bacteria between seasons in each type of wastewater (differences significant for $p < 0.05$). One-way ANOVA and a Scheffe's test (as the most conservative post hoc test, $p < 0.05$) were used to evaluate the differences between the percentages of resistance of *E. coli* isolates in the tested wastewater. The correlation between the number of tested microorganisms and the wastewater temperature was assessed using the Pearson correlation ($p < 0.05$). The tests were performed with Statistica v. 13.1 (StatSoft, Tulsa, OK, USA).

3. Results

Typically, the only monitoring tool used to assess the microbiological safety of reclaimed water is the total concentration of indicator organisms, including fecal coliforms (thermotolerant) and *E. coli*. Utilities and regulatory agencies rely on an assumed relationship between the indicator organism and pathogen survival/transport through wastewater treatment plants to ensure that the reclaimed water is safe for public use. While it is impossible to test reclaimed water for all possible pathogens, it is important that the indicator organisms used to ensure water quality correlate with a wide range of waterborne pathogens, such as bacteria, viruses, and protozoa.

Therefore, the number of fecal coliforms and *Escherichia coli*, including isolates resistant to antimicrobial agents, was determined as the basic indicator of bacterial contamination in sanitary analyses to assess the degree of contamination with fecal contamination.

In all analyzed wastewater samples, the presence of fecal coliforms, including *E. coli*, was found. Tables 2 and 3 compare of the concentrations of fecal coliforms and *E. coli* in the inflowing and treated wastewater. The amount of antibiotic-resistant *E. coli* isolated in the treated wastewater is presented in Table 4.

Table 2. The amount of fecal coliform bacteria in the inflowing and treated wastewater.

Sampling Date		Faecal Coliform Bacteria (FC) (CFU·100 cm^{-3})				The Degree of Reduction FC (%)	Average Temperature (°C)	
		Inflowing Wastewater		Treated Wastewater				
		The Amount of FC	Average Number of FC with SD	The Amount of FC	Average Number of FC with SD		Inflowing Wastewater	Treated Wastewater
Spring	Mar	3.7×10^5	$4.73 \pm 1.1 \times 10^5$ ab *	5.3×10^3	$7.13 \pm 2.1 \times 10^3$ a	98.5	13.70	14.00
	Apr	4.6×10^5		6.7×10^3				
	May	5.9×10^5		9.4×10^3				
Summer	Jun	6.1×10^5	$6.43 \pm 3.1 \times 10^5$ b	11.1×10^3	$13.53 \pm 2.6 \times 10^3$ b	97.9	18.30	18.30
	Jul	6.5×10^5		13.2×10^3				
	Aug	6.7×10^5		16.3×10^3				
Autumn	Sep	5.1×10^5	$4.70 \pm 4.6 \times 10^5$ ab	8.6×10^3	$5.57 \pm 2.6 \times 10^3$ a	98.8	14.70	15.30
	Oct	4.8×10^5		4.2×10^3				
	Nov	4.2×10^5		3.9×10^3				
Winter	Dec	3.8×10^5	$4.03 \pm 5.9 \times 10^5$ a	3.1×10^3	$3.03 \pm 0.1 \times 10^3$ a	99.3	9.70	10.00
	Jan	4.7×10^5		3.1×10^3				
	Feb	3.6×10^5		2.9×10^3				

* Averages marked with the same letters are not significantly different by Tukey test (α = 0.05) (one-way ANOVA, separately for each type of wastewater between seasons).

Table 3. The amount of *Escherichia coli* in the inflowing and treated wastewater.

Sampling Date		*Escherichia coli* (CFU·100 cm^{-3}) Inflowing Wastewater		Treated Wastewater		The Degree of Reduction *E. coli* (%)	Average Temperature (°C)	
		The Amount of *E. coli*	Average Number of *E. coli* with SD	The Amount of *E. coli*	Average Number of *E. coli* with SD		Inflowing Wastewater	Treated Wastewater
Spring	Mar	3.0×10^4	$3.77 \pm 0.9 \times 10^4$ a	44	59 ± 19.30 a	99.84	13.70	14.00
	Apr	3.5×10^4		53				
	May	4.8×10^4		81				
Summer	Jun	5.2×10^4	$5.23 \pm 0.2 \times 10^4$ b	94	111 ± 19.43 b	99.79	18.30	18.30
	Jul	5.1×10^4		106				
	Aug	5.4×10^4		132				
Autumn	Sep	4.2×10^4	$3.63 \pm 0.6 \times 10^4$ a	71	44 ± 24.27 a	99.88	14.70	15.30
	Oct	3.6×10^4		37				
	Nov	3.1×10^4		24				
Winter	Dec	2.9×10^4	$2.93 \pm 0.2 \times 10^4$ a	15	15 ± 1.53 a	99.95	9.70	10.00
	Jan	3.1×10^4		16				
	Feb	2.8×10^4		13				

Averages marked with the same letters are not significantly different by Tukey test (α = 0.05) (one-way ANOVA, separately for each type of wastewater between seasons).

Table 4. The amount of antibiotic-resistant *E. coli* isolated from treated wastewater.

Sampling Date		Total Amount of *E. coli* Isolates	Number of *E. coli* Isolates Resistant to:							
			ERY	AZM	CLR	OFX	CIP	AMP	TMP	MTR
Spring	March	44	17	11	6	7	8	23	27	0
	April	53	19	14	6	8	10	28	33	0
	May	81	31	20	9	12	16	44	50	0
Summer	June	94	45	48	34	28	27	64	58	0
	July	106	51	54	38	31	31	71	68	0
	August	132	64	68	48	39	39	89	116	0
Autumn	September	71	15	13	10	8	14	33	42	0
	October	37	8	8	6	4	7	18	22	0
	November	24	5	4	4	3	4	11	15	0
Winter	December	15	2	2	2	0	2	2	10	0
	January	16	3	3	3	5	3	3	14	0
	February	13	2	2	2	2	2	2	13	0

During the research, the microbiological quality of the inflowing wastewaters was different. The amount of thermotolerant fecal coliforms ranged from 4.03×10^5 to 6.43×10^5 CFU· 100 cm^{-3} (Table 2). *E. coli* ranged in the amount from 2.93×10^4 to 5.23×10^4 CFU· 100 cm^{-3} (Table 3). The number of fecal coliform bacteria in the treated wastewater ranged from 3.03×10^3 to 13.53×10^3 CFU· 100 cm^{-3}, with the lowest concentrations observed in the autumn and winter, and the highest in the spring and summer (Table 2). The number of *E. coli*, including antibiotic-resistant isolates, was varied (Tables 3 and 4). The summer and winter periods were characterized by the greatest variability in the concentrations of the analyzed indicators in the wastewater. This could be due to the share of runoff during sampling and the low temperature of the wastewater in winter.

Statistical analysis showed significant differences in the average number of fecal coliforms in the inflowing wastewater only during the summer and winter periods ($p < 0.05$). In the case of treated wastewater, there was a statistically significant difference in the average number of fecal coliforms between summer and other seasons of the year ($p < 0.05$). However, no significant differences were found between spring, autumn, and winter ($p > 0.05$) (Table 2).

With regard to *E. coli*, statistical analysis showed significant differences in the average number of these bacteria in incoming wastewater, between the summer season and other seasons of the year ($p < 0.05$). No significant differences were found between the spring, autumn and winter seasons ($p > 0.05$) (Table 3).

The question always arises whether the microorganism concentration in the inflowing wastewater affects the effectiveness of their removal in the biological treatment process, or whether it is primarily influenced by the design and operation of the treatment plant. In the conducted seasonal research series, the number of fecal coliforms and *E. coli* was reduced by over 99% (Tables 2 and 3) and the levels of these indicators were within the requirements of the permit. However, in the treated wastewater, as many as 89% (610) of isolates resistant to seven out of eight selected antibiotics were found among *E. coli* (Table 4). Only 11% (76) of the isolates were susceptible to the action of all tested antibiotics, which may be of concern (Figure 2). The most effective was metronidazole, to which 100% of the isolates showed susceptibility. The greatest numbers of isolates were resistant to trimethoprim (68%) and ampicillin (56%), while the least to the effect of ofloxacin—21%. A high percentage of resistant isolates was also found for erythromycin (38%) and azithromycin (36%) (Table 5). Resistance variability also depends on the season, as observed. The greatest numbers of antibiotic-resistant *E. coli* were isolated in the summer and the least in the winter. In August, among 132 isolates, as many as 116 were resistant to at least one tested antibiotic. In February, among the 13 tested isolates, all showed resistance to trimethoprim and only 2 to erythromycin, azithromycin, clarithromycin, ofloxacin, ciprofloxacin, and ampicillin (Table 4). In the conducted studies, resistance to as many as 7 antibiotics was simultaneously demonstrated by 14% of isolates. However, the number of multi-antibiotic-resistant strains to 4 or more antibiotics was significantly lower than those resistant to 1, 2, or 3 antibiotics (Figure 2).

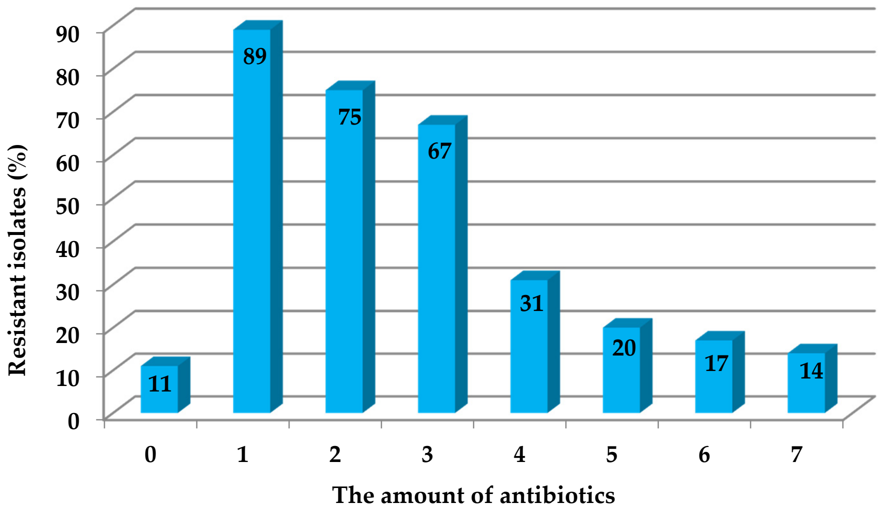

Figure 2. Percentage of multi-antibiotic-resistant *E. coli* isolates.

Table 5. Amount of antibiotic susceptible and resistant *E. coli* in the treated wastewater.

Antibiotic	Number of Susceptible Strains	Number of Resistant Strains	Resistance (%)
ERY	424	262	38
AZM	439	247	36
CLR	518	168	24
OFX	539	147	21
CIP	523	163	23
AMP	298	388	56
TMP	218	468	68
MTR	686	0	-

Taking into account the seasons, statistically significant differences were found between the percentages of *E. coli* resistance to antimicrobial agents. Significant seasonal differences were found for the antibiotics: erythromycin, azithromycin, clarithromycin, and ampicillin ($p < 0.05$). No significant differences between the percentages of resistance of *E. coli* isolates to antimicrobial agents in the seasons were observed to ofloxacin, ciprofloxacin, trimethoprim, and metronidazole ($p > 0.05$) (Table 4).

4. Discussion

The persistence of fecal coliforms and *E. coli* is related to the initial concentration of these microorganisms and the biological treatment effectiveness. Each subsequent stage of treatment can eliminate the concentration of indicators, depending on the characteristics of wastewater and key technological parameters. If the treatment system is highly effective, e.g., by using disinfection, the indicator concentrations may be below the detection limits [22–25].

Wastewater treatment plants operating on the basis of activated sludge technologies are characterized by different effectiveness in the elimination of sanitary indicators. In the conducted studies, the largest amount of coliform bacteria and *Escherichia coli* was observed in the inflowing sewage during the summer. However, in the process of biological treatment, their amount was reduced significantly above 90% in all research series. An equally high degree of reduction of fecal coliform bacteria was observed by Fars et al. [26].

Currently, more and more attention is paid to the participation of *E. coli* in the increase in antibiotic resistance in the aquatic environment. In the conducted studies, a total of 686 strains of *E. coli* were isolated from treated wastewater. The most common resistance was observed to trimethoprim (68%) and ampicillin (56%) (Table 5). Similar results were obtained by Piganto et al. [27] and Patoli et al. [28], considering ampicillin as the antibiotic to which *E. coli* bacteria isolated from water were the most resistant (88.89%; 22.71%; 18% resistant strains). In our research, a large percentage of resistant isolates was also found in the case of erythromycin and azithromycin. However, no resistance to metronidazole was observed (Table 5).

During the performed tests, the variability of resistance to antibiotics was slightly dependent on the season. The increase or decrease in resistance was according with the type of tested antibiotic, as observed (Tables 4 and 5). This phenomenon can be related to various interacting factors. The tested wastewater had a specific chemical composition. This was a mixture of municipal and industrial wastewater (1:2) and contained various inhibitory substances of organic and inorganic origin that can affect bacteria. These are referred to as stressors [29–31]. Examples of stressors are antibiotic residues and their degradation products, which can shape the surviving microbial community in the wastewater treatment process. Because of this, different organisms or related groups have different tolerance degrees or defense mechanisms [32,33]. Thus, depending on the type of antibiotic that may be present in the wastewater treatment plant, different bacterial resistance behavior in the treated wastewater was observed. For example, literature data show that metronidazole is active against coliform bacteria [34]. Therefore, in the conducted studies it was included in the evaluation of *E. coli* resistance. As expected, none of the *E. coli* isolates showed resistance in the tests performed. However, the occurrence of metronidazole-resistant strains in subsequent analyzes cannot be predicted. The observed difference in resistance may also be related to the microbiota (mainly composed of human commensal bacteria) present during the study, which is mixed with bacteria of various origins. These microorganisms can colonize the activated sludge [35], in which the fraction of antibiotic-resistant bacteria can reach much more than 50%, at least within a given group (e.g., *E. coli*), e.g., as a result of horizontal gene transfer [35,36].

In the conducted studies, 11% of *E. coli* isolates were susceptible to all tested antibiotics. Unfortunately, the remaining isolates (89%) were resistant to one or more antibiotics. Resistance to as many as 7 antibiotics was demonstrated simultaneously by 14% of isolates (Figure 2). Sahm et al. [37] found that strains multi-antibiotic resistant to 4 or 5 antibiotics are much less than those that are resistant to one or two, which was confirmed by the conducted studies.

The fractions of antibiotic-resistant bacteria present in the wastewater and the antibiotic resistance genes in the aeration reactor came into contact with the activated sludge microorganisms. When subjected to the potential selection pressure of antibiotics, the potential for the spread of antibiotic resistance increased. Microbiota (including antibiotic-resistant bacteria) reaching the biological reactor is stimulated to compete with the activated sludge bacteria for the available organic matter. This intense metabolic activity creates an important dynamic for the bacterial community [38]. Changes in the bacterial community, the performance of antibiotic-resistant bacteria, and the success with which their genes spread to other bacteria are the key factors important to the transmission of antibiotic resistance [39]. Because wastewater treatment plants are directly connected to the aquatic environment and are not adapted to eliminate antibiotic resistance genes and antibiotic-resistant bacteria, they promote their spread among enteric and pathogenic bacteria [40–42].

The evaluation of antibiotic resistance of *E. coli* isolates indicates the presence of antibiotic-resistant bacteria in treated wastewater, even with the high degree of reduction in sanitary indicators. This results in a release of it to the aquatic environment and the spread of resistant strains. The obtained results contribute to the increased awareness of antibiotic-resistant bacteria spread through wastewater treatment plants, despite the satisfactory removal of other indicators (BOD_5, COD, total nitrogen, total phosphorus, total suspended solids) [43]. In addition, they confirm the need for wastewater treatment techniques to prevent the antibiotic resistance spread, as recommended by the World Health Organization [44].

5. Conclusions

In the conducted studies, the concentrations of traditional bacterial indicators (fecal coliforms and *E. coli*) differed significantly according with the temporal wastewater samples. Despite almost 100% effectiveness of the biological treatment of wastewater in the elimination of fecal coliform bacteria, including *E. coli*, a fairly large percentage of *E. coli* isolates resistant to antimicrobial agents was found in the biologically treated wastewater. Perhaps in the process of wastewater disinfection, the number of strains carrying resistance genes into the water could be significantly reduced. From this study, it is evident that increased monitoring for alternative markers and pathogens is warranted in order to build a better database on the prevalence and reduction of intestinal bacteria, viruses, and parasites in reclaimed water. There should also be more discussion on the effectiveness of the process requirements compared to the reclaimed water quality targets.

The treated effluents monitored in WWTP in Oświęcim are not free from coliform bacteria, including antibiotic-resistant *E. coli*. This wastewater is discharged into the Vistula River, which means that public exposure to these waters carries some risk, although this level may be very low and quite acceptable for the majority of the population. Integrating microbiological monitoring with controls related to process design and operations can lead to a more robust approach to ensuring the safety of reclaimed water.

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References

1. Zhang, S.; Huang, J.; Zhao, Z.; Cao, Y.; Li, B. Hospital Wastewater as a Reservoir for Antibiotic Resistance Genes: A Meta-Analysis. *Public Health Front.* **2020**, *8*, 2296–2565. [CrossRef]
2. Luo, L.; Yao, J.; Liu, W.; Yang, L.; Li, H.; Liang, M.; Ma, H.; Liu, Z.; Chen, Y. Comparison of bacterial communities and antibiotic resistance genes in oxidation ditches and membrane bioreactors. *Sci. Rep.* **2021**, *11*, 8955. [CrossRef] [PubMed]
3. Alexander, J.; Hembach, N.; Schwartz, T. Evaluation of antibiotic resistance dissemination by wastewater treatment plant effluents with different catchment areas in Germany. *Sci. Rep.* **2020**, *10*, 8952. [CrossRef] [PubMed]
4. Rizzo, L.; Manaia, C.; Merlinc, C.; Schwartz, T.; Dagot, C.; Ploy, M.C.; Michael, I.; Fatta-Kassinos, D. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Sci. Total Environ.* **2013**, *447*, 345–360. [CrossRef] [PubMed]
5. Majeed, H.J.; Riquelme, M.V.; Davis, B.C.; Gupta, S.; Angeles, L.; Aga, D.S.; Garner, E.; Pruden, A.; Vikesland, P.J. Evaluation of Metagenomic-Enabled Antibiotic Resistance Surveillance at a Conventional Wastewater Treatment Plant. *Front. Microbiol.* **2021**, *12*, 1048. [CrossRef]
6. Barancheshme, F.; Munir, M. Strategies to Combat Antibiotic Resistance in the Wastewater Treatment Plants. *Front. Microbiol.* **2018**, *17*, 2603. [CrossRef]
7. Adefisoye, M.A.; Okoh, A.I. Identification and antimicrobial resistance prevalence of pathogenic *Escherichia coli* strains from treated wastewater effluents in Eastern Cape, South Africa. *Microbiologyopen* **2016**, *5*, 143–151. [CrossRef] [PubMed]
8. Praveenkumarreddy, Y.; Akiba, M.; Guruge, K.S.; Balakrishna, K.; Vandana, K.E.; Kumar, V. Occurrence of antimicrobial-resistant *Escherichia coli* in wastewater treatment plants of South India. *J. Water Sanit. Hyg. Dev.* **2020**, *10*, 48–55. [CrossRef]
9. Fouz, N.; Pangesti, K.N.A.; Yasir, M.; Al-Malki, A.L.; Azhar, E.I.; Hill-Cawthorne, G.A.; Abd El Ghany, M. The Contribution of Wastewater to the Transmission of Antimicrobial Resistance in the Environment: Implications of Mass Gathering Settings. *Trop. Med. Infect. Dis.* **2020**, *5*, 33. [CrossRef]
10. Karkman, A.; Do, T.T.; Walsh, F.; Virta, M.P.J. Antibiotic-Resistance Genes in Waste Water. *Trends Microbiol.* **2018**, *26*, 220–228. [CrossRef] [PubMed]
11. Watkinson, A.J.; Micalizzi, G.B.; Graham, G.M.; Bates, J.B.; Costanzo, S.D. Antibiotic-resistant *Escherichia coli* in wastewaters, surface waters, and oysters from an urban riverine system. *Appl. Environ. Microbiol.* **2007**, *73*, 5667–5670. [CrossRef] [PubMed]
12. Thakali, O.; Brooks, J.P.; Shahin, S.; Sherchan, S.P.; Haramoto, E. Removal of Antibiotic Resistance Genes at Two Conventional Wastewater Treatment Plants of Louisiana, USA. *Water* **2020**, *12*, 1729. [CrossRef]
13. Nguyen, A.Q.; Vu, H.P.; Nguyen, L.N.; Wang, Q.; Djordjevic, S.P.; Donner, E.; Yin, H.; Nghiem, L.D. Monitoring antibiotic resistance genes in wastewater treatment: Current strategies and future challenges. *Sci. Total Environ.* **2021**, *783*, 146964. [CrossRef]
14. Lucena, F.; Duran, A.; Morón, A.; Calderón, E.; Campos, C.; Gantzer, C.; Skraber, S.; Jofre, J. Reduction of bacterial indicators and bacteriophages infecting faecal bacteria in primary and secondary wastewater treatments. *J. Appl. Microbiol.* **2004**, *97*, 1069–1076. [CrossRef] [PubMed]
15. Aslan, A.; Cole, Z.; Bhattacharya, A.; Oyibo, O. Presence of Antibiotic-Resistant *Escherichia coli* in Wastewater Treatment Plant Effluents Utilized as Water Reuse for Irrigation. *Water* **2018**, *10*, 805. [CrossRef]
16. Wen, Q.; Tutuka, C.; Keegan, A.; Jin, B. Fate of pathogenic microorganisms and indicators in secondary activated sludge wastewater treatment plants. *J. Environ. Manag.* **2009**, *90*, 1442–1447. [CrossRef] [PubMed]
17. Barancheshme, F.; Munir, M. Development of Antibiotic Resistance in Wastewater Treatment Plants. In *Antimicrobial Resistance-A Global Threat*, 1st ed.; Kumar, Y., Ed.; IntechOpen Limited: London, UK, 2019; Available online: https://www.intechopen.com/books/antimicrobial-resistance-a-global-threat/development-of-antibiotic-resistance-in-wastewater-treatment-plants (accessed on 17 April 2021). [CrossRef]
18. EN ISO 9308-1:2000. Part 1: Membrane filtration method. In *Water Quality—Detection and Enumeration of Escherichia coli and coli form Bacteria*; American National Standards Institute (ANSI): New York, NY, USA, 2007.
19. Pepper, I.L.; Gerba, C.G. *Environmental Microbiology. A laboratory Manual*, 2nd ed.; Elsevier AP: Burlington, VT, USA, 2008; pp. 123–139.
20. CLSI. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 13th ed.; CLSI Standard M02; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2018; Available online: https://kaldur.landspitali.is/focal/gaedahandbaekur/gnhsykla.nsf/5e27f2e5a88c898e00256500003c98c2/94e1f81249a5e5560025756d005a560f/$FILE/M02Ed13E%20Performance%20Standards%20for%20Antimicrobial%20Disk%20Susceptibility%20Tests.pdf (accessed on 13 December 2019).
21. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 10.0. 2020. Available online: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_10.0_Breakpoint_Tables.pdf (accessed on 13 December 2019).
22. Paśmionka, I.B. Evaluation of the efficiency of removing sanitation indicators in the process of biological wastewater treatment. *Acta Sci. Pol. Formatio Circumiectus* **2020**, *19*, 15–22. [CrossRef]
23. Delanka-Pedige, H.M.K.; Munasinghe-Arachchige, S.P.; Zhang, Y.; Nirmalakhandan, N. Bacteria and virus reduction in secondary treatment: Potential for minimizing post disinfectant demand. *Water Res.* **2020**, *177*, 115802. [CrossRef]
24. Maffettone, R.; Manoli, K.; Santoro, D.; Passalacqua, K.D.; Wobus, C.E.; Sarathy, S. Performic Acid Disinfection of Municipal Secondary Effluent Wastewater: Inactivation of Murine Norovirus, Fecal Coliforms, and Enterococci. *Environ. Sci. Technol.* **2020**, *54*, 12761–12770. [CrossRef]

25. Boni, W.; Parrish, K.; Patil, S.; Fahrenfeld, N.L. Total coliform and *Escherichia coli* in microplastic biofilms grown in wastewater and inactivation by peracetic acid. *Water Environ. Res.* **2021**, *93*, 334–342. [CrossRef]
26. Fars, S.; Oufdou, K.; Nejmeddine, A.; Hassani, L.; Melloul, A.A.; Bousselhaj, K.; Amahmid, O.; Bouhoum, K.; Lakmichi, H.; Mezrioui, N. Antibiotic resistance and survival of faecal coliforms in activated sludge system in a semi-arid region (Beni Mellal, Morocco). *World J. Microbiol. Biotechnol.* **2005**, *21*, 493–500. [CrossRef]
27. Pignato, S.; Coniglio, M.A.; Faro, G.; Weill, F.X.; Giammanco, G. Plasmid-mediated multiple antibiotic resistances of *Escherichia coli* in crude and treated wastewaters used in agriculture. *J. Water Health* **2009**, *7*, 251–258. [CrossRef]
28. Patoli, A.A.; Patoli, B.B.; Mehraj, V. High prevalence of multi-drug resistant *Escherichia coli* in drinking water samples from Hyderabad. *Gomal J. Med. Sci.* **2010**, *8*, 23–26.
29. Barbosa, V.; Morais, M.; Silva, A.; Delerue-Matos, C.; Figueiredo, S.A.; Domingues, V.F. Comparison of antibiotic resistance in the influent and effluent of two wastewater treatment plants. *AIMS Environ. Sci.* **2021**, *8*, 101–116. [CrossRef]
30. Schages, L.; Wichern, F.; Geisen, S.; Kalscheuer, R.; Bockmühl, D. Distinct Resistomes and Microbial Communities of Soils, Wastewater Treatment Plants and Households Suggest Development of Antibiotic Resistances Due to Distinct Environmental Conditions in Each Environment. *Antibiotics* **2021**, *10*, 514. [CrossRef]
31. Cycoń, M.; Mrozik, A.; Piotrowska-Seget, Z. Antibiotics in the Soil Environment—Degradation and Their Impact on Microbial Activity and Diversity. *Front. Microbiol.* **2019**, *10*, 338. [CrossRef] [PubMed]
32. Pazda, M.; Kumirska, J.; Stepnowski, P.; Mulkiewicz, E. Antibiotic resistance genes identified in wastewater treatment plant systems—A review. *Sci. Total Environ.* **2019**, *697*, 134023. Available online: https://doi.org/10.1016/j.scitotenv.2019.134023 (accessed on 9 February 2021).
33. Lucassen, R.; Rehberg, L.; Heyden, M.; Bockmühl, D.P. Strong correlation of total phenotypic resistance of samples from household environments and the prevalence of class 1 integrons suggests for the use of the relative prevalence of intI1 as a screening tool for multi-resistance. *PLoS ONE* **2019**, *14*, e0218277.
34. Onderdonk, A.B.; Louie, T.J.; Tally, F.P.; Bartlett, J.G. Activity of metronidazole against escherichia coli in experimental infra-abdominal sepsis. *J. Antimicrob. Chemother.* **1979**, *5*, 201–210. [CrossRef] [PubMed]
35. Rabbia, V.; Bello-Toledo, H.; Jiménez, S.; Quezada, M.; Domínguez, M.; Vergara, L.; Gómez-Fuentes, C.; Calisto-Ulloa, N.; González-Acuña, D.; López, J.; et al. Antibiotic resistance in *Escherichia coli* strains isolated from Antarctic bird feces, water from inside a wastewater treatment plant, and seawater samples collected in the Antarctic Treaty area. *Polar Sci.* **2016**, *10*, 123–131. [CrossRef]
36. Frąc, M.; Jezierska-Tys, S.; Oszust, K.; Gryta, A.; Pastor, M. Assessment of microbiological and biochemical properties of dairy wastewater sludge. *Int. J. Environ. Sci. Technol.* **2017**, *14*, 679–688. [CrossRef]
37. Sahm, D.F.; Thornsberry, C.; Mayfield, D.C.; Jones, M.E.; Karlowsky, J.A. Multidrugresistant urinary tract isolates of *Escherichia coli*: Prevalence and patient demographics in the United States in 2000. *Antimicrob Agents Chemother.* **2001**, *45*, 1402–1406. [CrossRef] [PubMed]
38. Aghalari, Z.; Dahms, H.U.; Sillanpää, M.; Sosa-Hernandez, J.E.; Parra-Saldívar, R. Effectiveness of wastewater treatment systems in removing microbial agents: A systematic review. *Glob. Health* **2020**, *16*, 13. [CrossRef]
39. Barrios-Hernández, M.L.; Pronk, M.; Garcia, H.; Boersma, A.; Brdjanovic, D.; van Loosdrecht, M.C.M.; Hooijmans, C.M. Removal of bacterial and viral indicator organisms in full-scale aerobic granular sludge and conventional activated sludge systems. *Water Res. X* **2020**, *6*, 100040. [CrossRef]
40. Pazda, M.; Rybicka, M.; Stolte, S.; Piotr Bielawski, K.; Stepnowski, P.; Kumirska, J.; Wolecki, D.; Mulkiewicz, E. Identification of Selected Antibiotic Resistance Genes in Two Different Wastewater Treatment Plant Systems in Poland: A Preliminary Study. *Molecules* **2020**, *25*, 2851. [CrossRef]
41. Bengtsson-Palme, J.; Kristiansson, E.; Larsson, D.G.J. Environmental factors influencing the development and spread of antibiotic resistance. *FEMS Microbiol. Rev.* **2018**, *42*, fux053. [CrossRef] [PubMed]
42. Müller, H.; Sib, E.; Gajdiss, M.; Klanke, U.; Lenz-Plet, F.; Barabasch, V.; Albert, C.; Schallenberg, A.; Timm, C.; Zacharias, N.; et al. Dissemination of multi-resistant Gram-negative bacteria into German wastewater and surface waters. *FEMS Microbiol. Ecol.* **2018**, *94*, fiy057. [CrossRef]
43. World Health Organization. Briefing Note: Antimicrobial Resistance: An Emerging Water, Sanitation and Hygiene Issue. 2014. Available online: https://apps.who.int/iris/bitstream/handle/10665/204948/WHO_FWC_WSH_14.7_eng.pdf?sequence=1&isAllowed=y (accessed on 11 April 2021).
44. Jóźwiakowski, K.; Bugajski, P.; Mucha, Z.; Wójcik, W.; Jucherski, A.; Nastawny, M.; Siwiec, T.; Mazur, A.; Obroślak, R.; Gajewska, M. Reliability and efficiency of pollution removal during long-term operation of a one-stage constructed wetland system with horizontal flow. *Sep. Purif. Technol.* **2017**, *187*, 60–66. [CrossRef]