

## The Occurrence of Integrase Genes in Different Stages of Wastewater Treatment

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### ABSTRACT

The uncontrolled use of antibiotics and the release of drug residuals into the environment contribute to antibiotic resistance and constitute a serious public health threat. The spread of antibiotic resistance can be attributed mainly to the huge amounts of bacteria harboring the antibiotic resistance genes (ARGs) which are released into the environment with the treated wastewater. The molecular mechanisms of antibiotic resistance, in which the mobile genetic elements (MGEs) such as plasmids, transposons, bacteriophages and integrons associated with the transfer of ARGs play the main role, should be broadly investigated to develop effective methods for addressing this problem. This study focused mainly on integrons which: (i) are the simple elements involved in the mobility of gene cassettes, (ii) have a common structure, (iii) can be associated with other MGEs, and (iv) are particularly efficient in trapping ARGs. The aim of the study was to estimate the efficiency of different stages of the wastewater treatment process in removing class 1, 2 and 3 integrase genes in two wastewater treatment plants (WWTPs) in Poland and to investigate the presence of these genes in river water upstream and downstream from the effluent discharge point. The presence of *intI1*, *intI2* and *intI3* genes was analysed by means of standard PCR with specific primers and a thermal cycling profiles. The samples of wastewater and sludge were collected from two WWTPs located in the Polish regions of (a) Warmia and Mazury, and (b) Silesia. The samples of river water were also collected upstream and downstream from the examined WWTPs. In the selected WWTPs, wastewater is treated through the activated sludge process with various modifications. The presence of *intI1*, *intI2* and *intI3* genes in different stages of wastewater treatment was characterized by a similar pattern. The results of this study indicate that WWTPs are not highly effective in removing the integrase genes from all three integron classes. The study revealed somewhat unexpected results, which indicate that the recently discontinued monitoring of the *intI3* gene in the wastewater treatment process should be reinstated. The existing wastewater treatment systems should be improved and modified to effectively eliminate the integrase genes from wastewater and prevent contamination of the surface water.

**Keywords:** integrase, integron, ARGs, wastewater

### INTRODUCTION

Nowadays, antibiotic resistance is a global health issue in which the main role is played by the antibiotic resistant bacteria (ARB) that carry and transfer the antibiotic resistance genes (ARGs) into the environment [Pruden et al. 2013, Narciso–da-Rocha and Manaia 2016]. Antibiotic resistance is caused not only by the persistence

of the naturally occurring ARGs in bacteria or the evolution of ARGs in the bacterial communities colonizing the habitats characterized by high amounts of antibiotics and their transformation products (TPs) [Osińska et al., 2017a]. The spread of ARGs by horizontal gene transfer (HGT) and the rate at which this process occurs give a much greater cause for concern. Mobile genetic elements (MGEs) such as integrons, plasmids,

transposons, insertion sequences and genomic islands play an important role in incorporating new genes into the bacterial genome [Lekunberri et al., 2018]. Moreover, the widespread problem of antibiotic resistance is favored by the aquatic environment as a main pathway of pollutants spreading. Wastewater treatment plants (WWTPs) play an important role in this process as a major source of organic compounds and nutrients for bacterial proliferation [Makowska et al., 2016]. Raw wastewater contains carbon compounds as well as sub-inhibitory concentrations of antibiotics and other antimicrobials [Giebułtowicz et al., 2018] which promote the bacterial adaptation and contribute to the development of highly resistant bacteria [Rizzo et al., 2013]. High bacterial loads and the specificity of WWTPs promote the contact and gene transfer between microbial species or genera and between bacteria and the environment [Karkman et al., 2018]. The treated wastewater containing dangerous bacteria is often discharged to rivers, from where the antibiotic resistance determinants and ARB are transferred to other water bodies and environments, thus posing a considerable health risk for humans and animals [Paiva et al., 2015, Subirats et al., 2018].

Modern WWTPs deploy various wastewater treatment methods, including mechanical, biological and mechanical-biological methods with elevated removal of nutrients, sequencing batch reactors (SBR) or anaerobic/anoxic/oxic (A2 / O) systems [Korzeniewska and Harnisz 2018]. According to Rizzo et al. [2013], the biological treatment systems, where wastewater is continuously mixed, could be responsible for faster distribution of new genes. New treatment methods capable of removing a substantial percentage of pharmaceuticals from the treated wastewater have been developed over the past 20 years. However, these methods are very expensive, and there is still a demand for effective and low-cost treatment systems [Berglund et al. 2014]. Until such methods are deployed in WWTPs, highly virulent microorganisms will continue to be discharged with treated wastewater into the environment.

The essence of spreading antibiotic resistance in the environment should be sought in its molecular foundations. In this study, special attention was paid to integrons which are linked to MGEs. Integrons have been detected in the DNA of commensal and pathogenic bacteria colonizing humans and animals [Paiva et al., 2015]. Integrons used to be called a critical intermediate in

the capturing and expression process of resistance genes [Giebułtowicz et al., 2018]. They are able to build specific gene cassettes that carry ARGs, metal resistance genes, protein transport determinants and other proteins with unknown features into bacterial genome [Koczura et al., 2016]. In the group of the identified HGT mechanisms, conjugation significantly simplifies the transfer of integrons between bacteria via plasmids or transposons [Giebułtowicz et al., 2018]. Due to their specific abilities, integrons are used as indicators to determine the distribution patterns of different resistance genes in the bacterial population [Le et al., 2016].

The objective of this study was to estimate the efficiency of particular steps of the treatment process in removing the integron-integrase genes class 1, 2 and 3 in two WWTPs and to determine the distribution pattern of the integrase genes in the samples of river water and wastewater collected in different stages of the treatment process in summer and autumn. An attempt to estimate seasonal fluctuations in the content of *intI1*, *intI2* and *intI3* genes in wastewater was made as well.

## MATERIAL AND METHODS

### Characterization of WWTPs and samples collection

The study was conducted in two WWTPs located in the Polish regions of (a) Warmia and Mazury, and (b) Silesia. In the examined WWTPs, wastewater is treated through the activated sludge process with various modifications: (a) mechanical-biological methods with an elevated removal of nutrients (b) mechanical-biological treatment methods with sequencing batch reactor technology (C-TECH) (Table 1). The samples of wastewater, river water and sludge were collected in sterile bottles and urine containers, respectively, and they were transported to the laboratory under refrigerated conditions (4°C).

### Samples preparation and genomic DNA extraction

Specific amounts (Table 2) of the collected water and wastewater samples were passed through polycarbonate filters with 0.2 µm porosity (Merck, Millipore). The filters were transferred to sterile Falcon tubes for DNA extraction. The

**Table 1.** Characteristic of examined WWTPs

Location of wastewater treatment plant	Type of wastewater treatment system	Type of inflowing wastewater	Average processing capacity (m <sup>3</sup> /d)
Warmia and Mazury District	Mechanical-biological method with elevated removal of nutrients	Domestic sewage, hospital sewage, food industry sewage	35,000
Silesian District	Mechanical-biological method with sequencing C – TECH reactors	Domestic sewage, industry sewage	30,000

genomic DNA from water and wastewater samples was extracted with the DNeasy Power Water Kit (Qiagen). In order to obtain gDNA from sediments, 0.25 g of each sample was weighed and isolated with the DNeasy Power Soil Kit (Qiagen) according to the manufacturer's protocol. The quality and concentration of the extracted DNA were measured with the Multiskan Sky Microplate Spectrophotometer (Thermo Scientific). All extracted gDNA samples were stored at -20°C.

#### Detection of the integron – integrase genes by standard PCR

The class 1 (*intI1*), 2 (*intI2*) and 3 (*intI3*) integron-integrase genes were identified molecularly with standard PCR. The single PCR mixture of 20 µl contained the NZY Taq II 2x Green Master Mix, the DNA template and specific primers recommended by Goldstein (2001) and Dillon

(2005). Primer sequences and annealing temperature are given in Table 3. After the amplification of specific DNA fragments, all samples were visualized through electrophoresis in 1.5% agarose gel which was prepared by suspending agarose in 1 X TBE (Tris – Borate – EDTA) buffer and ethidium bromide staining (0.5 µg/mL). Electrophoresis was conducted at 120V/10 min and 80V/60 min.

## RESULTS AND DISCUSSION

Despite the fact that more than 99% of microorganisms are eliminated from wastewater during the technological processes in WWTPs [Osińska et al. 2017b, Korzeniewska and Harnisz 2018], alarming amounts of pathogenic bacteria are discharged to rivers and other water bodies with treated effluents [Ben et al.

**Table 2.** Type of collected samples and filtered amount

Type of sample	Place of sampling	Amount of filtered sample (mL)/sample weight (g)	
		Summer	Autumn
Untreated wastewater	WWTP in Warmia and Mazury District	40	40
Wastewater after the primary settling tank		40	40
Wastewater after the biological chamber		40	20
Wastewater after the multi – functional reactor		40	40
Treated wastewater		200	200
Sample from collector before the discharge of treated wastewater		200	400
Sample from collector after the discharge of treated wastewater		200	400
Sludge from the open fermentation pool		0.25	0.25
Sludge ready to development		0.25	0.25
Untreated wastewater		WWTP in Silesian District	10
Wastewater after the primary settling tank	30		30
Wastewater after the secondary settling tank	200		300
Wastewater after the selector and C – TECH reactor	150		300
Treated wastewater	150		300
Sample from collector before the discharge of treated wastewater	150		300
Sample from collector after the discharge of treated wastewater	150		300
Suspension outflowing after the dewatering process	10		30
Sludge after the mechanical compression	0.25		0.25
Sludge after the gravity compression	0.25		0.25

**Table 3.** Primer sequences and conditions of PCR.

Gene name	Primer sequence	Amplicon size [bp]	Annealing temperature [°C]	Reference
<i>intI1</i>	CCTCCCGCACGATGATC	280	55	Goldstein 2001
	TCCACGCATCGTCAGGC			
<i>intI2</i>	TTATTGCTGGGATTAGGC	233	50	
	ACGGCTACCCTCTGTTATC			
<i>intI3</i>	GCCTCCGGCAGCGACTTTTCAG	979	59	Dillon et al. 2005
	ACGGATCTGCCAACCTGACT			

2017, Munir et al. 2011]. Wastewater treatment plants are a specific highway to the rapid spread of antibiotic resistance by promoting the bacterial contact and enabling microorganisms to incorporate new genes into their genome as well as transport these genes to other bacteria through the HGT mechanism [Gatica et al. 2016, Guo et al. 2017]. The process of capturing and incorporating virulence genes would not be as effective without integrative and conjugative elements (ICEs) [Beceiro et al. 2013]. Integrons play an indisputable role in the spread of antibiotic resistance [Laroche et al. 2009], which is why many studies have investigated the presence of the integrase gene, an element responsible for catalyzing the recombination between the incorporated gene cassettes and the recombination site (*attI*) [Gillings 2014, Deng et al. 2015].

In this study, the class 1, 2 and 3 integron-integrase genes were detected in different stages of wastewater treatment. The class 1 integron-integrase genes were not effectively removed with the mechanical-biological methods with an elevated removal of nutrients in the WWTP in Warmia and Mazury or by means of the mechanical-biological methods with C-TECH reactors in the WWTP in Silesia (Tables 4 and 5). The removal efficiency of the bacteria carrying *intI1* genes was also low in the WWTPs investigated by Du et al. [2015] and Li et al. [2014]. The class 1 integrons are found mainly in Gram-negative bacteria such as *Acinetobacter*, *Alcaligenes*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Salmonella*, *Shigella*, *Staphylococcus* [Deng et al. 2015, Xu et al. 2011], some of which are common pathogens that cause numerous infections [Kaushik et al. 2019]. For this reason, the class 1 integron-integrase genes are often identified in WWTPs that process hospital wastewater. The previous studies have demonstrated that WWTPs act as hotspots for the spread of the pathogenic and antibiotic-resistant

bacteria, as well as that the released microorganisms and the carried genes can contribute to the spread of virulence in the environment [Calhau et al. 2015, Osińska et al. 2017b].

According to some authors, the class 2 integrons are less common than the class 1 integrons [Deng et al. 2015, Barlow and Gobius 2006]. In the present study, *intI2* were almost as prevalent as *intI1* in both WWTPs. Class 2 integrase is characteristic of the bacteria that colonize the human and animal feces (e.g. *E. coli* and *E. faecalis*); therefore, *intI2* is abundant in wastewater [Uyaguari et al. 2013]. It is also frequently carried by the bacteria of the *Acinetobacter*, *Salmonella* and *Pseudomonas* genera. The presence of the class 2 integron genes in the treated wastewater discharged into a river is highly alarming. In a study by Korzeniewska and Harnisz (2018), the percentage of ARB in total bacterial counts was much higher in the samples of treated wastewater than raw wastewater.

During the wastewater monitoring in WWTPs, the main emphasis is placed on the class 1 and 2 integrons. The class 3 integrons are far less prevalent, and they are monitored less frequently. However, Stadler et al. (2014) reported that the *intI3* gene was more abundant than the *intI2* gene in the wastewater samples collected from a WWTP. According to Uyaguari et al. (2013), the class 3 integrons are much more widespread in the environment than previously thought. Similar observations were made in the current study where *intI3* was present in the wastewater and sludge samples collected from both WWTPs. These results indicate that the monitoring of the *intI3* gene in the discharged effluents should be reinstated in WWTPs. Some authors also reported the presence of the class 1, 2 and 3 integrons in the untreated wastewater, in the wastewater samples collected in different stages of treatment, as well as in the treated wastewater [Moura et al. 2010, Uyaguari et al. 2013].

**Table 4.** Presence of the class 1–3 integrase genes in the samples from WWTP in the Warmia and Mazury District.

Type of sample	Season of sampling	Presence of genes		
		<i>intl1</i>	<i>intl2</i>	<i>intl3</i>
Untreated wastewater	Summer	+	+	+
Wastewater after the primary settling tank		+	+	+
Wastewater after the biological chamber		+	+	-
Wastewater after the multi – functional reactor		+	+	+
Treated wastewater		+	+	+
Sample from collector before the discharge of treated wastewater		+	-	+
Sample from collector after the discharge of treated wastewater		+	+	+
Sludge from the open fermentation pool		+	+	+
Sludge ready to development		+	+	+
Untreated wastewater		Autumn	+	+
Wastewater after the primary settling tank	+		+	+
Wastewater after the biological chamber	+		+	+
Wastewater after the multi – functional reactor	+		+	+
Treated wastewater	+		+	+
Sample from collector before the discharge of treated wastewater	+		-	+
Sample from collector after the discharge of treated wastewater	+		+	+
Sludge from the open fermentation pool	+		+	+
Sludge ready to development	+		+	+

**Table 5.** Presence of the class 1–3 integrase genes in the samples from WWTP in the Silesian District

Type of sample	Season of sampling	Presence of genes		
		<i>intl1</i>	<i>intl2</i>	<i>intl3</i>
Untreated wastewater	Summer	+	+	+
Wastewater after the primary settling tank		+	+	+
Wastewater after the secondary settling tank		+	+	+
Wastewater after the selector and C – TECH reactor		+	+	+
Treated wastewater		+	+	+
Sample from the collector before the discharge of treated wastewater		+	+	+
Sample from the collector after the discharge of treated wastewater		+	+	+
Suspension outflowing after the dewatering process		+	+	+
Sludge after the mechanical compression		+	+	+
Sludge after the gravity compression		+	+	+
Untreated wastewater	Autumn	+	+	+
Wastewater after the primary settling tank		+	+	+
Wastewater after the secondary settling tank		+	+	+
Wastewater after the selector and C – TECH reactor		+	+	+
Treated wastewater		+	+	+
Sample from the collector before the discharge of treated wastewater		+	+	+
Sample from the collector after the discharge of treated wastewater		+	+	+
Suspension outflowing after the dewatering process		+	+	+
Sludge after the mechanical compression		+	+	+
Sludge after the gravity compression		+	+	+

The results of this study indicate that the microbiological quality of the wastewater discharged to the surface waters is a serious concern that poses a considerable threat for the human and animal health. Further research to

develop new wastewater treatment methods or modify and combine the existing technologies to effectively reduce ARGs in the effluents discharged from WWTPs is recommended.

## CONCLUSION

Different types of wastewater that are treated in WWTPs with the use of various processing methods continue to pose a threat to humans and the environment. The treated wastewater containing significant amounts of potentially pathogenic microorganisms carrying the integrase genes may contribute to the growing levels of antibiotic resistance. The transmission of integrase genes between microorganisms during different stages of the wastewater treatment process may enhance the above-mentioned risk. Further work is recommended to modify the existing methods of wastewater treatment with a view to minimize the contamination of partially treated wastewater and reduce bacterial counts in the effluents discharged to the surface waters.

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