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Antibiotic Resistance Genes Dynamics at the Different Stages of the Biological Process in a Full-Scale Wastewater Treatment Plant †

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† Presented at the 3rd EWaS International Conference on "Insights on the Water-Energy-Food Nexus", Lefkada Island, Greece, 27–30 June 2018.

Published: 30 July 2018

Abstract: Wastewater treatment plants (WWTPs) highly contribute to the transmission of antibiotic resistance genes (ARGs) in the environment. In this work, the diversity of *ermF*, *ermB*, *sul1* and *int1*-enconding genes was examined in the influent, the mixed liquor and the effluent of a full-scale WWTP. Based on the clones analyzed, similar genotypes were recorded at all process stages. However, distinct genotypes of *int1* were responsible for the expression of *sul1* and *ermF* genes in *Gammaproteobacteria* and *Bacteroidetes*, respectively. Due to the detection of similar ARGs profiles throughout the biological process, it is concluded that additional treatment is needed for their retention.

Keywords: antibiotic resistance genes; microbiological effluent quality; biological processes; environmental monitoring; wastewater treatment

1. Introduction

The main source of antibiotics in the environment is the excretion of incompletely metabolized antibiotics by humans and animals. Thus, WWTPs are important reservoirs of antibiotic resistant bacteria, transmitting them into the aquatic ecosystems [1].

There are several studies examining the effectiveness of the different stages of the treating processes at reducing the concentrations of various antibiotics. Lin et al. [2] determined the effectiveness of primary and secondary treatment process, which was followed by a disinfection step, on the reduction of sulfonamides, cephalosporins, quinolones and macrolides. Batt et al. [3] studied the fate of four different antibiotics belonging to quinolones, sulfonamides and tetracyclines, during their treatment in four WWTPs, which differed in design and operating conditions. Moreover, Li and Zhang [4] and Watkinson et al. [5] recorded concentration removals within 25 and 80% during treatment of antibiotics in conventional treatment plants.

Despite that WWTPs are suitable for reducing the concentrations of pollutants in municipal wastewater, they appear to be insufficient in reducing antibiotic gene content [6]. By contrast, the activated sludge of municipal wastewater treatment plants is an ideal environment for the proliferation of antibiotic resistance genes since ARGs transmission can occur within the diverse microbial species of the flocs [7,8]. For instance, tetracycline, sulfonamide, macrolide and quinolone

resistance genes (*tet*, *sul*, *erm*, and *qnr*, respectively) have been detected in the treated effluent of WWTPs [9]. Moreover, Chen and Zhang [10] detected low number of copies of the sulfonamide antibiotic resistance genes *sul1* and *sul2*, and the integrase 1 gene (*int1*) in the effluent of certain WWTPs. The same authors reported a strong correlation within the amount of *sul1* and *int1* genes, indicating the involvement of integrase in *sul1* gene transmission [10].

Thus, the current study was conducted to investigate the distribution of antibiotic resistance genes (ARGs) in a sewage treatment plant, based on the molecular identification of three antibiotic resistance genes, i.e., *sul1*, *ermB* and *ermF*, and the integrase 1 gene (*int1*), which favors the spread of multiple genes to the microbial constituents of the activated sludge.

2. Materials and Methods

Samples from the influent, the mixed liquor and the effluent of WWTP were obtained by using sterile glass bottles. Genomic DNA extraction was conducted based on commercially available kit (Vivantis, Selangor Darul Ehsan, Malaysia). The macrolide (i.e., *ermB* and *ermF*) and the sulfonamide (i.e., *sul1*) resistance genes as well as the class 1 integron integrase (*int1*) genes were amplified by the use of the primer pairs erm(B)-454rc/erm(B)-91fc, erm(F)-189f/erm(F)-497r, sul1-F/sul1-B and int1-F/int1-R, respectively [11,12]. The amplification reaction was carried out in a TaKaRa Dice TP600 PCR thermocycler (Japan). The amplicons obtained were ligated into the bacterial vector pGEM-T Easy (Promega, Madison, WI, USA), and the recombinant DNA were transformed into DH5a competent cells. The plasmid DNA was extracted by the Vivantis plasmid kit (Malaysia) from the recombinant *Escherichia coli* cultures, and sequencing of the PCR inserts was performed at Macrogen using primers SP6 and T7 (Promega). The closest relatives of the amplicons obtained were subjected to BLAST searches. The amplified gene sequences and their closest relatives were translated to amino-acids by Emboss Transeq [13] and aligned by the Clustal Omega bioinformative tool [14].

3. Results and Discussion

A total of twelve clone libraries were constructed, each one for any antibiotic resistance gene and sampling point examined, i.e., the influent, the mixed liquor and the effluent of the full-scale WWTP. In the case of macrolides, the screening of *ermB* gene clone libraries resulted in the identification of three distinct genotypes. The major genotype consisting of 19 clones, which were identical to known sequences deposited in the International Sequencing database, while the second genotype comprised of 3 clones. Interestingly, the last genotype consisted of a single clone, which was only detected in the effluent of WWTP. On the other hand, the two major genotypes were detected throughout the whole biological treatment, a fact that indicates dispersion of this gene throughout the processing. The major *ermB* gene genotype showed high genetic relationship with respective genes carried out by strains of the genera *Streptococcus*, *Nocardia*, *Staphylococcus*, *Clostridium*, *Lactococcus* and *Listeria* (Table 1). Based on protein level analysis, the *ermB* genes detected were responsible for encoding a protein consisting of 107 amino acids, where similar gene translation patterns were identified. In comparison to other studies, Lee et al. [15] detected *ermB* genes in the effluent of two WWTPs, even after UV application as the disinfection method. Moreover, Wang et al. [6] showed the ineffectiveness of WWTPs in the removal of antimicrobial resistant genes, which were prevailing in all samples analyzed.

Regarding the detection of *ermF* genes in the WWTP examined, three distinct clone groups were identified, with the major clone cluster being consisted of 17 members. The *ermF* genes of the three genotypes were differentiated up to three nucleotide bases. It was also observed that the second group of *ermF* gene clones showed a close genetic relationship with the members of the first group and therefore, it could be considered as a subgroup of the dominant genotype. This led us to the conclusion that the dominant genotype of the *ermF* gene was detected throughout the biological treatment process. Although the third genotype consisted only of 3 clones, such genetic pattern was detected throughout the whole biological process. At protein level, only two distinct amino-acid patterns were identified as the result of the close relationship within the members of the first and the

second genotype. Almost all microorganisms carrying the *ermF* gene showed high genetic relation with respective genes detected only in members of the phylum *Bacteroidetes*, except of the gammaproteobacterium *Bibersteinia trehalosi* (Table 2). In comparison to other studies, Fahrenfeld et al. [16] detected *ermF* genes in the reclaimed water derived from a WWTP. Based on metagenomic analysis, Szczepanowski et al. [17] reported the prevalence of *ermF* gene in both activated sludge and the treated effluent of a WWTP.

Table 1. Identification of the major *ermB* genotypes in the WWTP examined.

Genotype	Gene Similarity	Microorganism Carrying the Closest Relative *ermB* Gene	GenBank Code
1	100%	*Streptococcus pneumoniae* ICESpnIC1	HG799494
		Nocardia farcinica CNM20080087	KM194594
		Streptococcus agalactiae GBS6	CP007572
		Staphylococcus aureus SA268	CP006630
		Clostridium difficile transposon Tn6218	HG002387
		Listeria monocytogenes LM78	JX535233
		Enterococcus faecium e82	JN899594
		Enterococcus faecalis plasmid pLG2	NG_041215
		Lactococcus garvieae plasmid pKL0018	AB290882
		Streptococcus uberis	EF540938
		Bacillus cereus 363	AF480455
		Streptococcus agalactiae KMP104	DQ355148
		Staphylococcus lentus	SLU35228
2	99%	*Streptococcus pneumoniae* NT_110_5	CP007593
		Streptococcus pyogenes HKU360	CP009612
		Enterococcus faecium Aus0085 plasmid p3	CP006623
		Streptococcus oligofermentans AS 1.3089	CP004409
		Streptococcus suis D12	CP002644
		Staphylococcus pseudintermedius C2597	JF909978
		Streptococcus uberis FSL Z3-097	EF539836
		Pediococcus acidilactici plasmid pEOC01	DQ220741
		Lactobacillus johnsonii G41 PEP-PTS	DQ518904
		Streptococcus cristatus transposon Tn6002	AY898750
		Streptococcus hyointestinalis	AY278215
		Lactobacillus fermentum	NG_034736
		Peptoclostridium difficile 630	CP010905
		Campylobacter jejuni C179b	KF864551
		Escherichia coli ECONIH1 plasmid pECO-824	CP009860
		Campylobacter coli SH-CCD11C365	KC876752
		Enterococcus thailandicus W3 plasmid pW3	NG_041564
		Lactobacillus plantarum plasmid pLFE1	FJ374272
3	99%	*Bacteroides uniformis* transposon WH207	AY345595
		Enterococcus faecium plasmid pXD5	KJ645709
		Staphylococcus hyicus plasmid pSTE1	HE662694
		Staphylococcus aureus SA7037 plasmid pV7037	NG_041616
		Enterococcus faecalis plasmid pTW9	AB563188
		Lactococcus garvieae plasmid pKL0018	AB290882
		Streptococcus suis 2-22	EU047808
		Streptococcus uberis FSL Z3-102	EF539835
		Arcanobacterium pyogenes	AY334073
		Staphylococcus intermedius MLS-17	AF239773
		Enterococcus hirae	AF406971
		Campylobacter jejuni C179b	KF864551

Regarding sulfonamides resistance genes, there was a dominant cluster consisting of 23 over 24 clones analyzed (carrying the *sul1* gene). The single representative of the second genotype possessed a distinct genetic position as compared to the dominant genotype, where genetic distance was greater than 4 base pairs. However, at protein level, its amino-acid sequence was similar with the major *sul1* cluster. Most of the microorganisms, which carried the *sul1* gene, exhibited high genetic similarity with *sul1* genes detected in strains of the class *Gammaproteobacteria* (Table 3). In the studies of Ben et al. [7] and Du et al. [18], *sul1* genes were the most abundant gene among the ARGs

examined in various WWTPs. Moreover, Lupan et al. [19] confirmed the spread of *sul1* genes in a river 10 km downstream of a WWTP.

Table 2. Identification of the major *ermF* genotypes in the WWTP examined.

Genotype	Gene Similarity	Microorganism Carrying the Closest Relative *Ermf* Gene	GenBank Code
1	100%	*Bacteroides ovatus* MN11	HE999703
2	100%	*Riemerella anatipestifer* RA-CH-1	CP003787
		Bacteroides salanitronis DSM 18170	CP002530
		Bibersteinia trehalosi USDA-ARS-USMARC-189	CP006955
		Barnesiella viscericola DSM 18177	CP007034
		Capnocytophaga sputigena Be58	JQ707297
		Bacteroides thetaiotaomicron transposon CTnDOT	AJ311171
3	97%	*Bacteroides salanitronis* DSM 18170	CP002530
		Bibersteinia trehalosi USDA-ARS-USMARC-189	CP006955
		Barnesiella viscericola DSM 18177	CP007034
		Bacteroides ovatus MN11	HE999703
		Capnocytophaga sputigena Be58	JQ707297
		Bacteroides thetaiotaomicron transposon CTnDOT	AJ311171

Table 3. Identification of the major *sul1* genotypes in the WWTP examined.

Genotype	Gene Similarity	Microorganism Carrying the Closest Relative *sul1* Gene	GenBank Code
1	100%	*Aeromonas hydrophila* AL06-06	CP010947
		Vibrio parahaemolyticus V36 plasmid pVPH1	KP688397
		Acinetobacter baumannii AB_NCGM 346	LC030435
		Escherichia coli 6409 plasmid p6409	CP010373
		Pseudomonas aeruginosa NCGM257	AP014651
		Klebsiella pneumoniae ATCC BAA-2146 plasmid pNDM-US-2	KJ588779
		Serratia marcescens 11663 plasmid p11663	AP014611
		Salmonella enterica plasmid pSBLT	LN794247
		Vibrio cholerae plasmid pRJ354C	KP076293
		Proteus mirabilis PEL	KF856624
		Enterobacter cloacae 34983 plasmid p34983	CP010378
		Pantoea sp. PSNIH1 plasmid pPSP-a3e	CP009883
		Proteus mirabilis PmC162	KJ186154
		Stenotrophomonas maltophilia GZP-Sm1	KM649682
		Klebsiella oxytoca MS5279 plasmid pKOI-34	AB715422
		Aeromonas salmonicida 2004-05MF26 plasmid pSN254b	KJ909290
		Nocardia nova CNM20121076	KM194585
2	99%	*Aeromonas hydrophila* AL06-06	CP010947
		Vibrio parahaemolyticus V36 plasmid pVPH1	KP688397
		Acinetobacter baumannii AB_NCGM 346	LC030435
		Escherichia coli O157:H16 strain Santai	CP007592
		Pseudomonas aeruginosa NCGM257	AP014651
		Klebsiella pneumoniae ATCC BAA-2146 plasmid pNDM-US-2	KJ588779
		Serratia marcescens 11663 plasmid p11663	AP014611
		Salmonella enterica plasmid pSBLT	LN794247
		Vibrio cholerae plasmid pRJ354C	KP076293
		Proteus mirabilis PmCHE	KJ439039
		Stenotrophomonas maltophilia GZP-Sm1	KM649682
		Klebsiella oxytoca MS5279 plasmid pKOI-34	AB715422
		Aeromonas salmonicida 2004-05MF26 plasmid pSN254b	KJ909290
		Nocardia nova CNM20121076	KM194585

Considering the genetic analysis of integrase class 1 gene, a prevalent clone cluster was detected, which included 25 out of 26 clones analyzed. Nevertheless, at protein level, these integrase class 1 clusters differed by a single amino acid. Interestingly, the microbial species of the major genotype carrying the *int1* gene were related to those carrying *sul1* genes. On the other hand, the

bacterial strain possessing the *int1* gene of the minor cluster was associated with microbiota carrying *ermF* genes (Table 4). A strong correlation between the concentrations of *sul1* and *int1* genes have been recently reported [6,7,18], which demonstrates the importance of integrons in the spread of these antibiotic resistance genes in the environment, indicating that *sul1* gene is frequently located on class 1 integron.

Table 4. Identification of the major *int1*genotypes in the WWTP examined.

Genotype	Gene Similarity	Microorganism Carrying the Closest Relative *int1* Gene	GenBank Code
1	100%	*Aeromonas hydrophila* sAL06-06	CP010947
		Klebsiella pneumoniae Kpn-3002cz plasmid pS-300cz	KJ958927
		Vibrio parahaemolyticus V36 plasmid pVPH1	KP688397
		Acinetobacter baumannii	LC030435
		Escherichia coli 6409 plasmid p6409	CP010373
		Pseudomonas aeruginosa NCGM257	AP014651
		Achromobacter xylosoxidans A22732 plasmid pA22732-IMP	KJ588780
		Klebsiella pneumoniae ATCC BAA-2146 plasmid pNDM-US-2	KJ588779
		Serratia marcescens 11663 plasmid p11663	AP014611
		Salmonella enterica plasmid incHI2	LN794248
		Acinetobacter baumannii A1	CP010781
		Vibrio cholerae plasmid pRJ354C	KP076293
		Proteus mirabilis PEL	KF856624
		Enterobacter cloacae 34983 plasmid p34983	CP010378
		Serratia marcescens A4Y201 plasmid pG5A4Y201	KJ541069
		Klebsiella oxytoca MS5279 plasmid pKOI-34	AB715422
		Nocardia veterana CNM20120791	KM194583
		Shigella flexneri Shi06HN006	CP004057
		Proteus mirabilis PmCHE	KJ439039
		Klebsiella pneumoniae blaNDM-1 plasmid 1	CP009116
2	99%	*Bacteroides salanitronis* DSM 18170	CP002530
		Bibersteinia trehalosi USDA-ARS-USMARC-189	CP006955
		Barnesiella viscericola DSM 18177	CP007034
		Bacteroides ovatus MN11	HE999703
		Bibersteinia trehalosi USDA-ARS-USMARC-192	CP003745
		Capnocytophaga sputigena Be58	JQ707297
		Bacteroides thetaiotaomicron transposon CTnDOT	AJ311171

4. Conclusions

Based on the molecular determination of *sul1*, *ermB*, *ermF* and *int1* genes, it is concluded that antibiotic resistant genes spread occurred throughout the biologic process in WWTPs. The similar genotyping profiles detected in the influent and the effluent point out the necessity for the implementation of effective tertiary treatment methods, regarding gene removal from the treated effluent. Further experiments on the use of advanced oxidation systems and membrane technologies will elucidate issues regarding the removal of antibiotic resistance genes. Moreover, different class 1 integrons appeared to be responsible for the transmission of *sul1* and *ermF* genes among members of distinct phyla, a fact that indicates a microbial specificity in antibiotic resistant gene transmission.

Author Contributions: I.Z. and I.A. performed the experiments; I.Z., S.N., P.M., I.A. and M.P. analyzed the data; I.Z. and S.N. wrote the paper; S.N. conceived and designed the experiments.

Conflicts of Interest: The authors declare no conflict of interest.

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