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Antibiotic resistance gene profiles at various treatment stages of a full-scale municipal sewage plant

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ABSTRACT

The discharge of wastewater treatment plants (WWTPs) is the main route for the transmission of antibiotic resistance genes (ARGs) in the aquatic environments. In this work, the diversity of *ermF*, *ermB*, *sul1* and *int1* genes were investigated at the various stages of the biological treatment process in a full-scale municipal sewage plant, that is, in the influent, the mixed liquor and the treated effluent of the WWTP examined. Application of culture-independent molecular techniques resulted in the detection of similar genotype patterns throughout the entire treatment process. In addition, evidence that distinct *int1* genotypes are responsible for the expression of *sul1* and *ermF* genes in members of *Gammaproteobacteria* and *Bacteroidetes* respectively indicates possible microbe specificity at phylum level. The identification of similar ARGs patterns throughout the biological treatment process also denotes the necessity for the implementation of effective tertiary treatment methods other than chlorination and ultraviolet disinfection to diminish their dissemination threat.

Keywords: Antibiotic resistance genes; Microbiological effluent quality; Biological process; Environmental surveillance

1. Introduction

Antibiotics have been widely used for the curation of infectious diseases in humans and animals [1]. However, only a small portion of such antimicrobial agents can be metabolized by humans or animals, thus the remaining part is excreted and released into the environment as parent compounds or metabolites via the urine and the faeces [2]. This is the way how the antibiotics can enter wastewater treatment plants (WWTPs) and sequentially discharge to recipient water bodies, like rivers, lakes and groundwater, due to their inefficient reduction during the implementation of conventional wastewater treatment methods [3].

There are several studies examining the effectiveness of WWTPs to reduce antibiotic concentrations at the various stages of the treatment process. Lin et al. [4] evaluated the effectiveness of primary and secondary treatment process, successively followed by a disinfection step, on the reduction of sulfonamides, cephalosporins, quinolones and macrolides. Batt et al. [5] examined the fate of antibiotics belonging to quinolones, sulfonamides and tetracyclines in full-scale WWTPs, reporting antibiotics removal efficiencies of 58%–98% when sand filtration and chlorination in series was applied as the tertiary treatment method. Moreover, Li and Zhang [6] found only 25% antibiotics removal efficiency during treatment of sewage in conventional treatment plants.

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By contrast, Watkinson et al. [7] reported antibiotics removal efficiencies even greater than 80% during municipal wastewater processing in conventional WWTPs.

The antibiotics occurrence, even at low concentrations into water bodies, apart from inducing toxicity on aquatic and terrestrial life, can favor the transmission of antibiotic resistance genes (ARGs) in such habitats [3]. Therefore, the dispersion of antibiotics in the natural environment can contribute to the development of antibiotic resistance, which was denoted by the World Health Organization as an increasingly serious threat to global public health [8].

In particular, the dissemination of ARGs is favored in WWTPs since the latter consist the main reservoir of antibiotic resistant bacteria and ARGs to aquatic environment [9]. Activated sludge and anaerobic digestion processes provide the ideal environment for the proliferation of ARGs, since their transmission can occur within diverse microbial species [10], even from non-pathogenic into pathogenic microbiota or among phylogenetically distant organisms through the horizontal gene transfer (HGT) mechanism occurred inside the flocs [3,11,12]. Indeed, ARGs can even increase in various processing steps carried out in WWTPs, resulting in elevated threat for the aquatic life [3].

HGT is the main molecular mechanism for ARGs dispersal across species, mediated by mobile genetic elements, like plasmids, transposons and integron associated gene cassettes, which play a major role in the short-term acclimatization of bacteria to increased antibiotic concentration and in their evolution over prolonged time period. Conjugation, transformation and transduction are the main ways of achieving HGT [10,13]. Regarding integrons, these are genetic elements capable of embedding ARGs within gene cassettes [14]. The dense and diverse microbial population in activated sludge proliferate ARGs transfer through plasmid conjugation among the microbial constituents of mixed liquor flocs [13].

A comprehensive study on the fate of ARGs and their transmission mechanisms in activated sludge systems can permit the understanding of their main dispersal routes into the environment. In the recent years, attempts have been performed to investigate the occurrence and predominance of various ARGs at the different stages of biological processing in full-scale sewage treatment systems. However, contradictory results have been extracted, since some researchers have reported that activated sludge treatment can result in reducing ARGs dissemination, whereas others have reported the opposite [15]. Besides, other scientific reports have recommended the inclusion of additional wastewater treatment steps, such as adsorption, membrane filtration and advanced oxidation processes, in order to effectively remove antibiotics and ARGs from the treated effluent [16]. Application of ultraviolet (UV), ozonation and chlorination has been used as disinfection strategies to diminish antibiotic-resistant bacteria and their ARGs into the recipient water bodies [17–19].

Several ARGs types conferring resistance to beta-lactams, macrolides, quinolones, sulfonamides and tetracyclines have been detected in the treated effluent of full-scale sewage treatment plants [9]. In particular, macrolide, quinolone, sulfonamide and tetracycline resistance genes (*erm*, *qnr*, *sul* and *tet* respectively) have been detected in the effluent of such biological treatment systems [20]. For example, Chen and Zhang [21] reported the occurrence of the sulfonamide ARGs *sul1* and *sul2*, as well as the integrase 1 gene (*int1*) in the effluent of several sewage plants. A strong correlation between the copy numbers of *sul1* and *int1* genes has been also found, denoting the involvement of integrase in *sul1* gene transmission mechanism [21].

Thus, this study aims at investigating the distribution of ARGs at the various treatment stages of a sewage treatment plant, based on the molecular identification of three ARGs, that is, *ermB*, *ermF* and *sul1*, representing resistance to commonly used antibiotics, as well as of one genetic indicator of the HGT, the class 1 integron gene (*int1*).

2. Materials and methods

2.1. Sampling procedure and deoxyribonucleic acid extraction

Samples were collected from the influent, the mixed liquor and the effluent of a full-scale sewage treatment plant by using autoclaved glass bottles [22]. The physicochemical characteristics of the influent and the effluent of the WWTP examined were determined according to Clesceri et al. [23] (Table 1).

A commercially available kit (Vivantis, Malaysia) was employed for extracting deoxyribonucleic acid (DNA) from samples obtained from the various stages of the biological process. Per each sampling point, duplicate samples were filtered through 0.45 μm membrane filters and the retained (on the membrane) biomass were subjected to DNA extraction.

2.2. Amplification of ARGs

The ARGs-examined were amplified from duplicate DNA samples and the polymerase chain reaction (PCR) products were pooled for clone library construction. Blanks were included in all PCR reactions. The macrolide resistance genes *ermB* and *ermF* were amplified by using the erm(B)-454rc (5′-GAA TCG AGA CTT GAG TGT GC-3′) and erm(B)-91fc (5′-GAT ACC GTT TAC GAA ATT GG-3′) as well as the erm(F)-189f (5′-CGA CAC AGC TTT GGT TGA AC-3′) and erm(F)-497r (5′-GGA CCT ACC TCA TAG ACA AG-3′) primer sets [24], respectively. Amplification of the sulfonamide resistance gene (*sul1*) was carried out by the primer set sul1-F (5′-CGG CGT GGG CTA CCT GAA CG-3′) and sul1-B

Table 1
Physicochemical characteristics in the influent and effluent of the Wastewater Treatment Plant examined

Parameter	Influent	Effluent
pH	7.79 ± 0.21	7.73 ± 0.07
EC (μS/cm)	1,212 ± 110	907 ± 26
SS (mg L^{-1})	237 ± 54	6.55 ± 2.77
VSS (mg L^{-1})	197 ± 67	5.31 ± 2.29
Total COD (mg L^{-1})	522 ± 116	24.53 ± 3.85
Soluble COD (mg L^{-1})	225 ± 78	14.93 ± 2.13
BOD_5 (mg L^{-1})	324 ± 76	18.33 ± 0.87
TKN (mg L^{-1})	99 ± 21	1.31 ± 0.49
NH_4^+–N (mg L^{-1})	67 ± 9.9	0.75 ± 0.34

(5'-GCC GAT CGC GTG AAG TTC CG-3'), whereas the class 1 integron gene (*int1*) was amplified by using the primers int1-F (5'-CCT CCC GCA CGA TGA TC-3') and int1-R (5'-TCC ACG CAC TGT CAG GC-3') [25]. The amplification reactions of the ARGs examined were performed in a Dice TP600 PCR thermocycler (TaKaRa, Japan) by preparing a PCR mixture of 20 ng genomic DNA, 10x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, the appropriate primers at concentration 0.5 mM each and 2.5 U DNA Taq polymerase (Kapa Biosystems, Wilmington, Massachusetts, USA). For the *ermB* and *ermF* genes, the amplification reaction included a denaturation stage of 2 min at 94°C, and 35 cycles comprising of a denaturation procedure of 30 s at 94°C, 30 s primer annealing at 52°C or 54°C, respectively and DNA fragment elongation at 72°C for 45 s. For the *sul1* and *int1* genes, the amplification reaction consisted of 2 min denaturation process at 94°C, and 35 thermocycles of 30 s denaturation at 94°C, 30 s primers' annealing at 60°C and 1 min DNA fragment elongation at 72°C. All the above-mentioned PCR reactions were terminated by an additional thermal step at 72°C for 7 min.

2.3. Construction of ARG clone libraries

The amplified ARGs from the various stages of the biological processes were ligated into the plasmid vector pGEM-T Easy (Promega, USA), using T4 DNA ligase (TaKaRa, Japan). The obtained recombinant plasmids were transformed into *Escherichia coli* DH5a competent cells. The plasmid DNA from the recombinant *E. coli* cultures was extracted by the "Vivantis plasmid kit" (Malaysia) and their PCR inserts were sequenced at Macrogen by using the vector primers SP6 and T7 (Promega, USA). After assembling the ARG amplicons in "CAP3 Sequence Assembly Program" [26], the similarity of the sequenced clones to their closest ARGs was identified by using the blastn option at National Center for Biotechnology Information (NCBI) database. Alignments of ARG amplicons sequenced in the current study were performed by using the "Clustal Omega" platform [27]. The construction of the phylogenetic trees was conducted by using the MEGA7 software [28] based on the application of the Jukes and Cantor algorithm [29]. The tree topology was inferred by the "neighbor-joining" method of Saitou and Nei [30] through 1,000-trees bootstrap support. The amplified ARGs were translated into amino-acids by the use of the web Emboss Transeq program (https://www.ebi.ac.uk/Tools/st/emboss_transeq/) and then aligned by the Clustal Omega tool [27]. MEGA7 for windows was employed for tree construction of the predicted peptides [28]. The numbers on tree nodes denote % bootstrap support based on 1,000 replicates. ARGs clone library coverages were calculated according to Magurran [31].

3. Results and discussion

A total of twelve clone libraries regarding the ARGs *ermB*, *ermF*, *sul1* and *int1* were constructed, corresponding to the various sampling points examined, that is, the influent, the mixed liquor and the effluent of a full-scale sewage treatment plant. In particular, a number of 102 clones were sequenced, that is, 23, 29, 24 and 26 *ermB*-, *ermF*-, *sul1*- and *int1*-gene containing clones, respectively. Their clone library coverages are presented in Table 2.

Table 2
Coverage of ARGs-clone libraries constructed

ARG	Coverage (%)		
	Influent	Mixed liquor	Effluent
ermB	100	100	87.5
ermF	90	87.5	90
sul1	100	100	90
int1	100	88.9	100

Regarding the macrolide resistance genes, the sequence of *ermB* gene-containing clone libraries resulted in the identification of three distinct genotypes (Fig. 1a).

The major genotype comprised of 19 clones, where 9 were identical to known ARG sequences reported in the NCBI, whereas the other clones of this group differed only by one or two nucleotide bases. The *ermB* genes of the predominant clone cluster showed a high genetic relationship (99.4%–100%) with respective genes carried out by strains of the genera *Streptococcus*, *Nocardia*, *Staphylococcus*, *Clostridium*, *Lactococcus* and *Listeria* (Table 3).

Even though Gram-negative bacteria, mainly *Proteobacteria*, are the main constituents of activated sludge [32], Di Cesare et al. [20] found that *ermB* genes were mainly hosted by Gram-positive bacteria, which is also the case in the current study. This might be a possible reason for the reduced occurrence of *ermB* genes during the biological treatment processes since Gram-negative have been found to be favoured over Gram-positive bacteria during biological treatment in WWTPs [33,34]. Considering the predominant genotype, this ARG group was detected throughout the biological treatment, denoting its dispersion to the recipient water body. This is in accordance to the findings of Yang et al. [35], who stated that the majority of ARGs are transferred from the influent to the activated sludge flocs during wastewater treatment. Moreover, the second genotype consisted of 3 clones, which were detected only in the influent and the effluent of the sewage plant. The fact that was not detected in the mixed liquor indicates low proliferation within the activated sludge constituents (Fig. 1a). The last genotype comprised of a single clone (OUT1ERMB), which was only identified in the treated effluent of the sewage plant (Fig. 1a), denoting possible ARGs transmission among settlement tank microbiota. Based on protein prediction analysis, the *ermB* genes detected were responsible for the encoding of a protein consisting of 107 amino acids, where similar gene translation patterns were identified. However, a distinct amino acid sequence in the case of clone ML2ERMB was predicted, which was clearly differed from those of the predicted *ermB* peptides belonging to the predominant cluster (Fig. 1b).

Similar to the findings of Wang et al. [36], the detection of the same *ermB* gene pattern throughout the biological process is indicative of the ineffectiveness of sewage treatment plant to eliminate the threat of transmitting *ermB* genes to the aquatic environment, even if application of disinfection methods, such as chlorination and UV light, occurred [20,37]. Recent findings have shown that chlorination appears to cause enrichment of *ermB* genes [38].

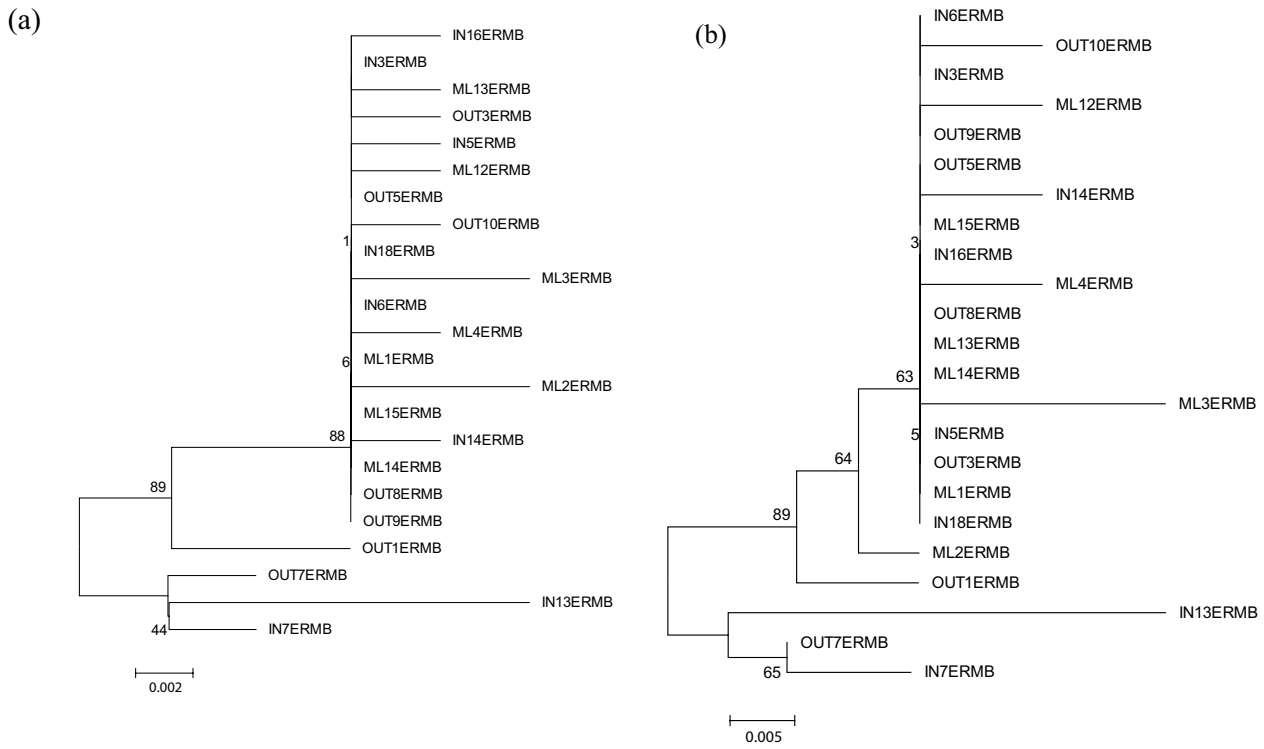

Fig. 1. Distribution of *ermB* genes (a) and their predicted peptides and (b) across the various treatment stages of the full-scale municipal sewage plant examined.

Three clone clusters were identified during the investigation of the fate of *ermF* genes, which is considered as the most prevalent macrolide resistance gene in the bacteria of the activated sludge systems [39]. The main *ermF* gene cluster comprised of 17 clones, whereas the remaining two clusters consisted of 9 and 3 clones. All the *ermF* genes that were placed in three genotypes were differed by up to three nucleotide pairs. Genetic analysis also revealed that the clones containing the *ermF* gene of the second cluster were closely related to the respective clones of the major *ermF* gene clone cluster (Fig. 2a).

Thus, the second cluster can be considered as a subgroup of the predominant genotype, indicating that the major *ermF* genotype was detected at all stages of the biological treatment process. Despite that the third genotype consisted only of 3 clones, its occurrence was observed throughout the biological treatment process (Fig. 2a). Similar to our study, Szczepanowski et al. [40], by employing metagenomic approaches, showed the occurrence of the same *ermF* genotype in the mixed liquor and the effluent of a full-scale WWTP. Moreover, Fahrenfeld et al. [41] reported the detection of *ermF* genes even in the reclaimed water of a sewage treatment plant.

At protein level, only two distinct *ermF* peptides were predicted as a consequence of the close relatedness of the first and the second genotype, providing further evidence that the second clone cluster is a subgroup of the major *ermF* genotype (Fig. 2b). All *ermF* genes detected in the current study showed high genetic similarity with *ermF* genes that were found mainly in members of the phylum *Bacteroidetes*, with the exception of *Bibersteinia trehalosi* (*Pasteurellaceae*, *Pasteurellales*, *Gammaproteobacteria*) (Table 4). Indeed, *Bacteroidetes* species commonly include *ermF* genes [42,43]. Interestingly, *Bacteroidetes*, which is included among the subdominant phyla of the activated sludge appears to resist chlorination [34,44].

Regarding sulfonamides resistance genes, the predominant genotype was comprised of 23 out of the 24 *sul1* gene-containing clones analyzed, which were detected at all stages of the biological treatment process (influent, mixed liquor and effluent) (Fig. 3a). The only clone of the second genotype (OUT6SUL1) was placed in a distinct genetic position in comparison to the predominant genotype since *sul1* gene divergence was greater than 4 nucleotide pairs (Fig. 3a). However, at protein level, the predicted *sul1*-encoded peptide was structurally similar to that of the major *sul1* cluster (Fig. 3b).

Exceptionally, the predicted *sul1*-encoded peptide of clone ML10SUL1, which was placed in the major genotype at gene level, was differed in amino-acid sequence from the other respective peptides (Fig. 3b). Almost all *sul1* genes sequenced in the current study showed high genetic similarity with *sul1* genes detected in bacteria that belong to the class *Gammaproteobacteria*, such as *Acinetobacter*, *Aeromonas*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Pantoea*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia*, *Stenotrophomonas* and *Vibrio*, with the only exception of those identified in *Nocardia* spp. (Table 5).

Sulfonamide resistance genes (*sul1*) have been detected in the effluents of several WWTPs in Italy and in the United States [20,45]. Ben et al. [11] and Du et al. [46] found that *sul1*

Table 3
Similarity of *ermB* genes detected in the current study with their closest *ermB* genes found in known microorganisms

Cluster (representative clone)	Similarity (in *ermB* gene)	Microorganism carrying the closest *ermB* gene	GenBank
Cluster 1 (IN3ERMB)	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
Cluster 2 (OUT1ERMB)	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
Cluster 3 (IN7ERMB)	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

genes were the most abundant ARGs in several WWTPs. Lee et al. [37] examined the dissemination of sulfonamide resistance genes in two WWTPs. A reduction of *sul* gene copies was observed in a WWTP after the biological treatment and the application of UV disinfection, whereas an increase in the number of *sul* gene copies was determined in another sewage plant. Interestingly, Lupan et al. [47] reported the dispersal of *sul1* genes even 10 km downstream the recipient water body.

Considering the diversity of class 1 integron gene, a prevalent genotype was identified, which was comprised of

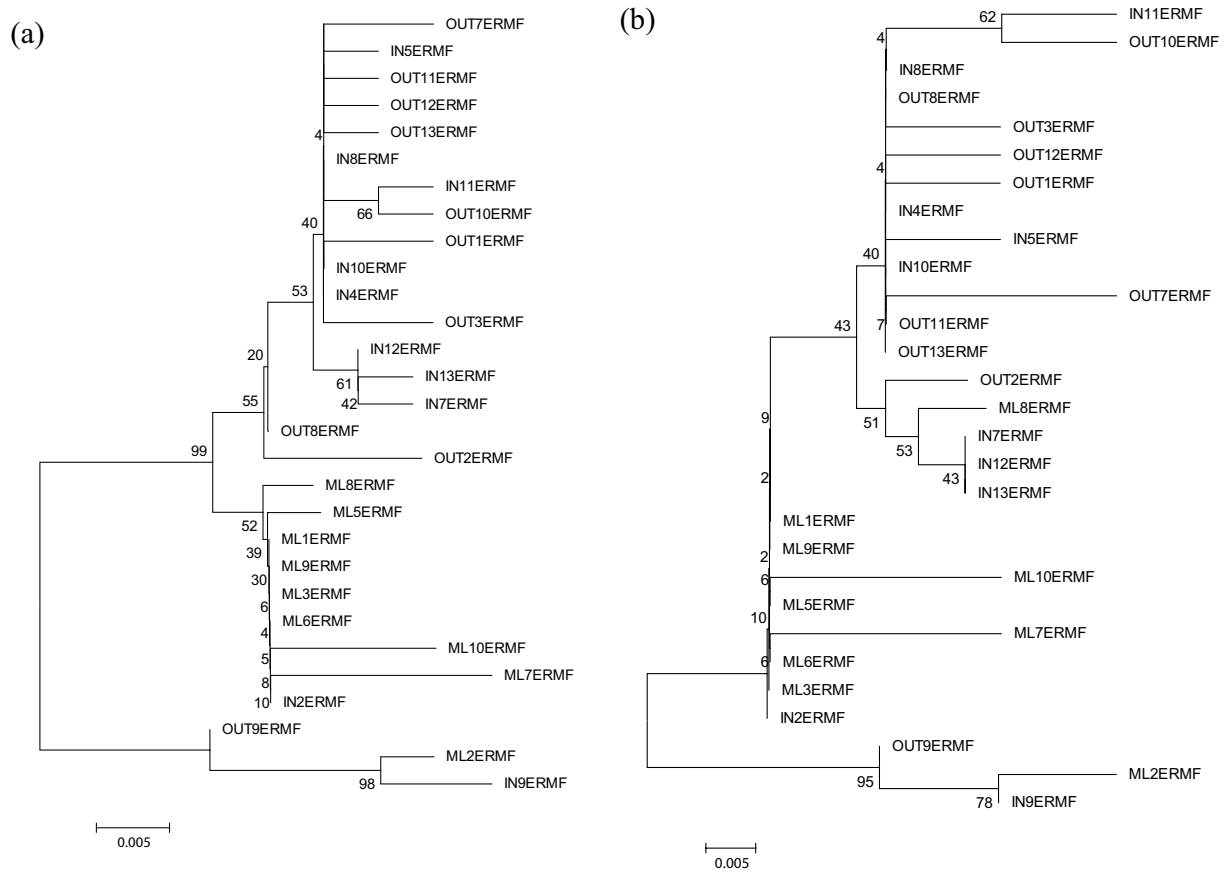

Fig. 2. Distribution of *ermF* genes (a) and their predicted peptides and (b) across the various treatment stages of the full-scale municipal sewage plant examined.

Table 4
Similarity of *ermF* genes detected in the current study with their closest *ermF* genes found in known microorganisms

Cluster (representative clone)	Similarity (in *ermF* gene)	Microorganism carrying the closest *ermF* gene	GenBank
Cluster 1 (IN8ERMF)	100%	*Bacteroides ovatus* MN11	HE999703
		Riemerella anatipestifer RA-CH-1	CP003787
		Bacteroides salanitronis DSM 18170	CP002530
Cluster 2 (ML9ERMF)	100%	*Bibersteinia trehalosi* USDA-ARS-USMARC-189	CP006955
		Barnesiella viscericola DSM 18177	CP007034
		Capnocytophaga sputigena Be58	JQ707297
		Bacteroides thetaiotaomicron transposon CTnDOT	AJ311171
Cluster 3 (OUT9ERMF)	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

25 out of the 26 clones sequenced (Fig. 4a). The only exception was the clone ML9INT1, which was placed in a distinct genetic position (Fig. 4a). However, at protein level, apart from the diverse amino acid sequence predicted for the clone ML9INT1, the predicted structure of integrase in the case of clone IN3INT1 also differed (by a single amino acid) from that of the other *int1*-containing clones of the major genotype (Fig. 4b).

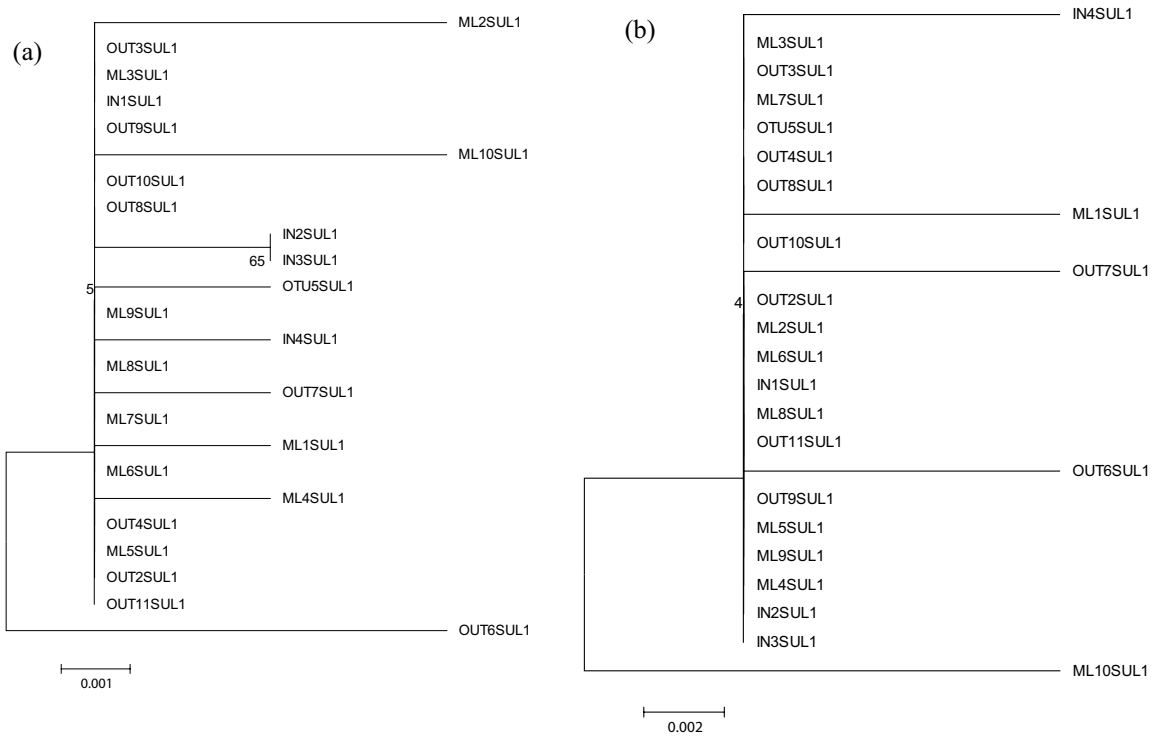

Fig. 3. Distribution of *sul1* genes (a) and their predicted peptides and (b) across the various treatment stages of the full-scale municipal sewage plant examined.

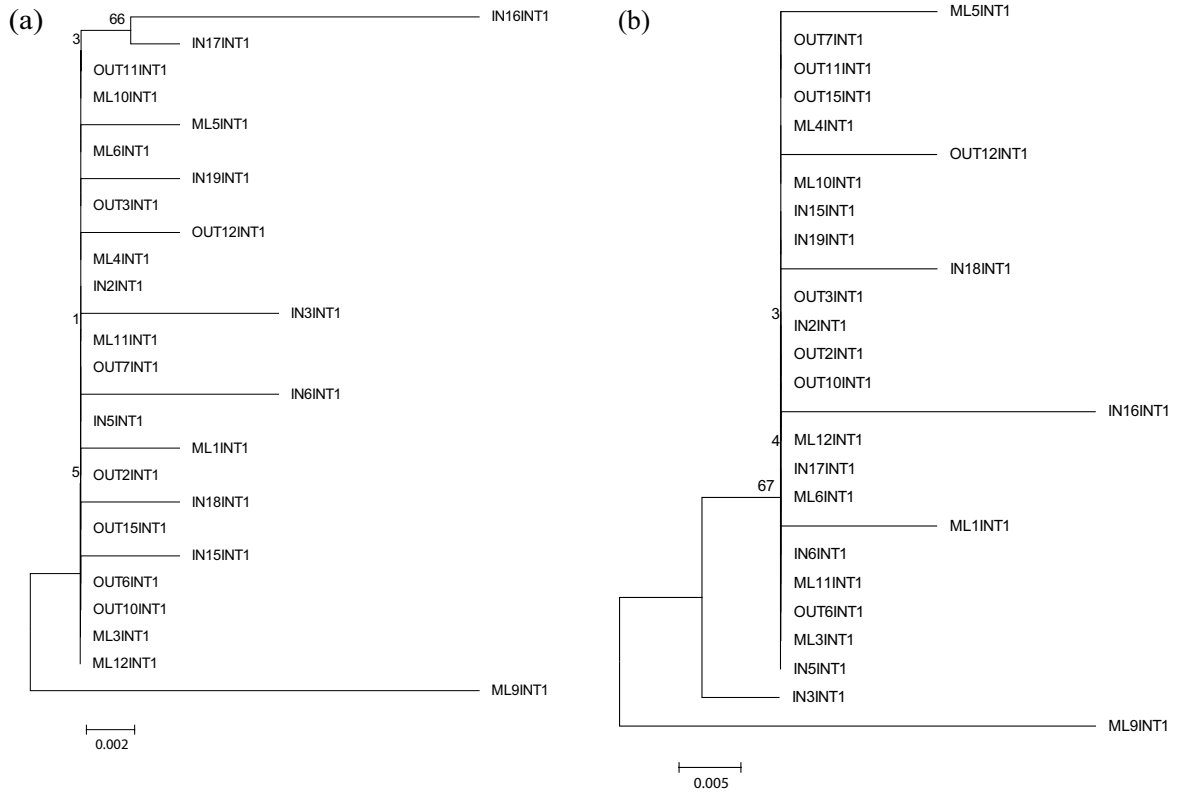

Fig. 4. Distribution of *int1* genes (a) and their predicted peptides and (b) across the various treatment stages of the full-scale municipal sewage plant examined.

Table 5
Similarity of *sul1* genes detected in the current study with their closest *sul1* genes found in known microorganisms

Cluster (representative clone)	Similarity (in *sul1* gene)	Microorganism carrying the closest *sul1* gene	GenBank
Cluster 1 (OUT3SUL1)	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
Cluster 2 (ML10SUL1)	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

Interestingly, the microorganisms, which their *int1* genes were closely related to the *int1*-containing clones of the major genotype identified in the current study, included *sul1* genes, which were also related to those detected in the present work (Table 6).

On the other hand, the bacterial strains possessing *int1* genes related to the single clone of the minor *int1* genotype included *ermF* genes similar to those identified in the current study (Table 6). A strong relationship between the abundance of *sul1* and *int1* genes have been found [11,36,46], a fact that denotes the involvement of integrons in the dispersal of sulfonamide resistance genes in the environment. In particular, *sul1* gene has been reported to be part of class 1 integron [39], where, herewith, such relationship was preferably found among members of the *Gammaproteobacteria* (Tables 5 and 6). On the other hand, a connection within *ermF* and *int1* genes appeared to be occurred, indicating possible inclusion of *ermF* gene on class 1 integron of the *Bacteroidetes* representatives that were present in the activated sludge of the WWTP examined (Tables 4 and 6). Thus, this indicates microbe specificity in the transmission of *sul1* and *ermF* genes in the environment.

4. Conclusions

Investigation of *sul1*, *ermB*, *ermF* and *int1* gene diversity in the full-scale WWTP examined resulted in the detection of ARGs throughout the biological treatment process. The similar genotype patterns detected in the influent and the effluent of the WWTP denotes the necessity of applying effective tertiary treatment methods, focusing on the reduction of both antibiotics and ARGs prior to effluent discharge in the recipient water bodies. Further research on the application of advanced oxidation processes and membrane technologies as well as on their economic feasibility will elucidate the efficiency of such treatment systems to diminish ARGs in the aquatic habitats. In addition, different class 1 integron gene appeared to be responsible for the

Table 6
Similarity of *int1* genes detected in the current study with their closest *int1* genes found in known microorganisms

Cluster (representative clone)	Similarity (in *int1* gene)	Microorganism carrying the closest *int1* gene	GenBank
Cluster 1 (OUT11INT1)	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
Cluster 2 (ML9INT1)	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

dissemination of *sul1* and *ermF* genes among strains of distinct bacterial phyla, a fact that indicates microbe specificity in ARGs 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.

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