Deliverable of WG4

Deliverable 16

Understanding the contribution of biological processes to antibiotic resistance spread into the environment

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CONTENTS

1. Introduction

As the potential function of treated wastewater reuse as an alternative source of water supply is now well acknowledged and embedded within international, European and national strategies, there have been concerted efforts which specifically target a substantial increase in recycling and safe reuse globally by 2030 (UN Sustainable Development Goal on Water, 2015). Likewise, wastewater reuse has been set as of uppermost significance in the Strategic Implementation Plan of the European Innovation Partnership on Water to deal with water scarcity in arid areas around the world (European Commission, 2012). Consequently, WasteWater Treatment Plants (WWTPs) offer a highly promising source of recycled water to deal with issues of water scarcity and water quality deterioration in problematic areas. However, the observation of the presence of microcontaminants of emerging concern (CECs) in treated wastewater effluents makes the task of wastewater reuse a challenging one.

The abundance of antibiotics potentially due to their overuse, misuse and incomplete removal by WWTP processes, accompanied by the presence of antibiotic-resistant bacteria (ARB) and their associated antibiotic resistance encoding genes (ARGs) has been witnessed in the last decades in treated wastewater effluents (Marti et al., 2013; Rizzo et al., 2013; Berendonk et al., 2015). Their presence has been well established in wastewater (Baquero et al., 2008; Czekalski et al., 2012), suggesting the significance of these aquatic matrices as potential pathways of human exposure to ARB (Allen et al., 2010). It has been established that non-pathogenic bacteria such as comensal bacteria may act as sinks of ARGs to pathogenic, clinically-relevant bacteria (Miller et al., 2016). Furthermore, treated wastewater used for irrigation has also been shown to be implicated in the transmission of water-borne pathogenic bacteria, which has led to the investigation of rigorous controls on the discharged effluent microbiological quality intended for irrigation, in various countries (Pachepsky et al., 2012; Al-Jassim et al., 2015). Such an example was given in the study by Al-Jassim et al. (2015), where a quantitative microbial risk assessment (QMRA) was conducted in order to determine the risk of infection by pathogenic bacteria associated with treated wastewater reuse in agricultural irrigation, in Saudi Arabia. The QMRA affirmed that chlorinated effluent use should be permitted for agricultural irrigation as it produced an acceptable annual microbial risk of lower than 10^{-4} arising from the opportunistic pathogens *Pseudomonas aeruginosa* and *Aeromonas hydrophila*. However, despite the improvement in microbial risk regarding the total counts of the abovementioned cultivable pathogens, there was an increase in relative resistance prevalence in

isolates to various classes of antibiotics (ampicillin, kanamycin, erythromycin, tetracycline, ceftazidime, ciprofloxacin, chloramphenicol, meropenem) from 3.8% in the influent to 6.9% in the effluent, highlighting the potential risk associated with treated wastewater reuse not only from conventional faecal indicators and known pathogens in general, but also from ARB&ARGs.

The presence of ARGs, which may be encoded in chromosomes or in extrachromosomal plasmids, can elicit biochemical defense mechanisms that allow the survival of bacteria in the presence of the corresponding antibiotic compounds such as in the case of treated wastewater, thus making them increasingly resilient in conditions of environmental stress. Also, plasmid-elicited resistance genes bear the potential of attainment by suitable bacteria through the horizontal gene transfer (HGT) mechanisms of conjugation, transformation and transduction (Yomoda et. al., 2003). Conjugative transfer mechanisms are highly common in natural environments where there is a high density of metabolically active bacterial cells which have a close proximity to each other, making the exchange of genetic material possible (Barkay et al., 1995), as different plasmid transfer mechanisms from donor to recipient cells have been described (Schwartz et al., 2003; Gomez-Alvarez et al., 2012).

Conventional WWTPs whose operation is typically based on biological treatment such as the conventional activated sludge (CAS) process, have been indicated as imperative hubs of ARB development and dissemination (Rizzo et al., 2013, Bouki et al., 2013). As conventional WWTP contain a high, nutrient-rich and a microbially dense biomass, they present ideal environments for the persistence of ARGs in activated sludge, wastewater effluents as well as in biosolids used as soil manure (Miller et al. 2016). The lack of inclusion of ARB&ARGs in existing regulations regarding treated wastewater reuse worldwide, poses a potential risk of microbiological contamination of ground and surface water, wildlife and food chains, as studies have shown their inadequate removal during biological treatment, releasing ARB&ARGs into environmental compartments (Becerra-Castro et al. 2015).

As a result, 'active stewardship' must be applied in wastewater treatment practices in order to prevent the selection pressure, flow and spread of AR determinants such as antibiotic residues, ARB&ARGs to and from environmental reservoirs (Pruden et al. 2013). The first and uttermost step to making this approach a reality, is the deep and thorough understanding of the contribution of biological wastewater treatment processes to antibiotic resistance spread into the environment through the

comprehensive review of the available scientific literature, with the ultimate aim of the optimization of wastewater treatment strategies, for the elimination of such microcontaminants of emerging concern (CEC) from urban wastewater influents, including ARB&ARGs. An important limitation currently imposing itself on a review of the available studies on reduction of ARB&ARGs during biological wastewater treatment processes is the discrepancy in methodological approaches followed in each study. More specifically, different experimental methodologies have been followed by different studies, making comparison of their findings difficult or even impossible. Moreover, the discrepancy between the use of cultivation methods examining a single or various cultivable bacteria, and molecular approaches such as PCR/qPCR/metagenomic methodologies, makes the comparison between studies and the draw of meaningful conclusions difficult and prone to errors. As a result, care has been taken in this review, to ensure the gathering of common methodologies and their associated findings, together when making conclusions in the different sections of this review. Next, the studies included in this review have been divided into four major sections, each regarding a different aspect of the investigation of the contribution of biological processes to antibiotic resistance spread in the environment. The most important conclusions arising in each of the abovementioned sections, are given as section sub-titles, followed by a discussion of the main studies present to provide evidence and support them. Consequently, studies are grouped together according to their aim of study, in each of the abovementioned sections.

These sections concern the following major aspects of biological wastewater treatment process removal of ARB&ARGs:

1) *Fate of ARB&ARGs during biological wastewater treatment processes*
2) *Impacts of biological treatment processes on the receiving environments*
3) *Sources and carriers of ARB&ARGs*
4) *Selective pressures and pressure conditions on ARB&ARGs during biological wastewater treatment.*

2. Comprehensive review of most recent studies to establish the contribution of biological processes to AR spread in the environment

2.1. Fate of ARB&ARGs during biological wastewater treatment processes

Various studies have examined the fate of ARB&ARGs during the municipal wastewater treatment (WWTP) process (Table 1). The findings of the studies indicate the change in cultivable and non-cultivable fractions of the bacterial communities, through the employment of cultivation and/or molecular techniques, respectively. The raw and treated effluent ARB&ARGs absolute or relative concentrations define the ability of the biological processes to reduce or remove these microcontaminants, in order to prevent their release into environmental compartments and subsequent spread and proliferation. The main findings of the most recent studies regarding the fate of ARB&ARGs during biological WWTP processes are given next.

Table 1 - Comprehensive review of the studies examining the fate of ARB&ARGs during biological wastewater treatment.

Refere-nce	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experime-ntal methods	Study findings
				FATE OF ARB&ARG DURING BIOLOGICAL WASTEWATER TREATMENT					
Osińska et al. (2017)	1. Determination of the frequency and distribution of antibiotic resistance determinants in *E. coli* strains isolated from untreated and treated WW and from river water; 2. Determination of the virulence and clonal relatedness of *E. coli* strains and the genetic fingerprints of selected *E. coli* strains.	Mechanical, biological, and chemical treatment followed by sludge processing.	Olsztyn, Poland.	amoxicillin, tetracycline and ciprofloxacin-resistant *E. coli* and clonal relatedness of isolates.	**Physicochemical parameters:** pH, temperature, BOD, COD.	**ARGs**: *aac(6')-Ib-cr, qnrD, qnrS, blaCTX-M, blaSHV, blaTEM, blaOXA, tetA, tetB, tetC, tetD, tetE, tetK, tetL, tetM, tetO, tetS, tetQ, tetX, tetA(P), Intl1, Intl2.* **Virulence genes**: *eae, bfpA,* CVD432, LT gene, ST gene, ipaH, *stx1, stx*2.	Total genomic DNA.	Antimicrobial susceptibility of isolates obtained on mFC agar, ERIC-PCR, conventional PCR.	1. The highest counts of beta-lactam, tetracycline and fluoroquinolone-resistant *E. coli* were found in untreated WW; 2. 317 isolates resistant to at least one group of antibiotics were selected for antibiotic susceptibility testing, showing that 38% of those isolates were resistant to all tested antibiotics. 3. 43% of multi-drug resistant isolates were found in untreated WW. Beta-lactam resistant isolates most often carried *blaTEM* and *blaOXA* genes; 4. The most encountered tetracycline resistance-encoding ARGs were *tetA*, *tetB* and *tetK*; 5. The virulence genes *bfpA* (65%), ST (56%) and *eae* were most widely distributed in examined isolates; 6. Multi-drug resistance was more often found in isolates from downstream river water than in isolates from upstream river water.

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colspan 10: **FATE OF ARB&ARG DURING BIOLOGICAL WASTEWATER TREATMENT**

Refere-nce	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experime-ntal methods	Study findings
Conte et al. (2017)	1. The evaluation of the occurrence of beta-lactam and quinolone resistance in *E. coli*, *Klebsiella pneumoniae* and *K. oxytoca*; 2. Assessment of the presence of ciprofloxacin residues in hospital sewage, WWTP and river water samples.	Urban WWTP and 640-bed tertiary care-teaching hospital.	Iguacu river and Curitiba region, Brazil.	ESBL-positive, carbapenem and quinolone-resistant *E. coli, K. pneumoniae, K. oxytoca*.	**Antibiotics:** Ciprofloxacin	**ARGs**: *blaTEM, blaSHV, blaCTX-M, blaGES, blaPER, blaKPC, blaNDM, blaVIM, blaSIM, blaGIM, blaBES, blaVEB, blaSPM, blaIMP, blaOXA, qnrA, qnrB, qnrC, qnrD, qnrS, qnrVC, qepA, oxqAB, and aac(6')-Ib-cr. gyrA* mutations.	NA	Antimicrobial susceptibility of isolates using VITEK-2 system, ERIC-PCR, conventional PCR, amplicon sequencing, Nucleotide and protein sequencing using Lasergene software package.	1. ESBL-producing isolates were found in raw and treated water samples, including *E. coli, K. pneumoniae* and *K. oxytoca*; 2. Cephalosporin and quinolone resistance was found in 34.4% of *E. coli* and 27.3% of *K. pneumonae*; 3. Carbapenem resistance was found in 5.4% of *K. pneumoniae* and *K. oxytoca*; 4. The presence of *blaCTX-M* (92.7%) and *blaSHV* (14.5) as well as of *blaGES* (3.6%) was established; 5. All sites contained quinolone resistance genes, except inflow sand aeration tanks; 6. Quinolone resistance was attributed to amino acid substitutions in the quinolone resistance determining regions of *gyrA* (47%) or the presence of plasmid-mediated quinolone resistance determinants; 7. Ciprofloxacin residues were absent only in upstream river water.

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Ben et al. (2016)	1. Quantification of release of antibiotic resistance from ten WWTP effluents; 2. Determination of correlation of antibiotic resistance with antibiotics and integrons in effluent; 3. Clarification of the effect of treatment processes on the elimination of antibiotic resistance in effluents.	1. Anaerobic/anoxic/oxic (A^2/O)-MBR-O^3; 2. Oxidation ditch-coagulation/sedimentration-UV; 3. anaerobic/oxic-MBR-UV; 4. A^2/O-ultrafiltration-O^3; 5. A/O-biofilter-ultrafiltration-UV; 6. A/O-O^3; 7. oxidation ditch-rotary fiber disc filter-UV; 8. A^2/O-rotary fiber disc filter-UV; 9. A^2/O-coagulation/sedimentation-rotary fiber disc filter-NaOCl; 10. A^2/O coagulation/sedimentation-rotary fiber disc filter-UV.	Beijing, Wuxi and Qingdao, China.	Tetracycline and sulfamethoxazole-resistant heterotrophic plate counts.	**Antibiotics:** *Sulfonamides:* sulfadiazine, sulfathiazole, sulfamerazine, sulfamethazine, sulfa-methizole, sulfamethoxazole, and sulfadimethoxine *Tetracyclines:* Tetracycline, oxytetracycline, chlortetracycline and doxycycline.	**ARGs:** *tetA, tetC, tetG, tetM, tetW, tetO, tetX, sul1, sul2, intl1, intl2*	Total DNA	Isolates on non-selective medium, qPCR.	1. The concentrations of ARB and ARGs ranged from 1.1×10^1 to 8.9×10^3 CFU mL^{-1} and 3.6×10^1 (*tetW*) to 5.4×10^6 (*tetX*) copies mL^{-1}, respectively; 2. The correlations between the ARB and ARGs concentrations with those of the antibiotics were not statistically significant; 3. However, there was a strong statistical correlation between total concentration of tetracycline resistance genes and sulfonamide resistance genes with *Intl1* concentrations; 4. The statistical analysis of the ARGs concentration in the effluents revealed the important role of disinfection in the eradication of antibiotic resistance.

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FATE OF ARB&ARG DURING BIOLOGICAL WASTEWATER TREATMENT									
Denden Rafraf et al. (2016)	1. Assessment of the removal efficiency of WWTP processes and evaluation of their potential to contribute to the dissemination of ARGs into the environment through measuring concentrations of beta-lactam, fluoroquinolone, macrolide and sulfonamide ARGs and of *intl1* as a proxy for gene transfer and anthropogenic pollution.	All 5 examined WWTPs undergo preliminary treatment to remove large solids. Three out of 5 WWTP provide secondary treatment through activated sludge with different capacities. One WWTP uses activated sludge followed by UV disinfection and another WWTP uses aerated lagoons as secondary process.	Monastir, Tunisia.	-	-	**ARGs:** *blaTEM, blaCTX-M, qnrA, qnrS, ermB, sul1, intl1.*	Total DNA.	qPCR.	1. All ARGs and the *intl1* gene were detected except the *blaCTX-M* gene, in influent and effluent samples from 2 WWTPs examined; 2. Although relative concentration of ARGs was similar in samples collected before and after treatment, the abundance of *blaCTX-M, blaTEM* and *qnrS* relative concentration was higher in effluent of the WWTP which receives domestic, industrial and untreated hospital waste.
Zanotto et al. (2016)	1. Detection of ampicillin-resistant and chloramphenicol-resistant coliforms and *Escherichia coli* in the inflow and outflow from a municipal WWTP.	Primary treatment: screening, sand and oil removal. Secondary treatment: activated sludge, including pre-denitrification and biological oxidation follow by rapid sand filtration and peracetic acid disinfection.	Milan, Italy.	Total coliforms, total *E. coli* and ampicillin and chloramphenicol-resistant *E. coli*.	-	**ARGs:** *Tem-1, catA1.*	ampicillin and chloramphenicol-resistant *E. coli* isolates.	Conventional PCR.	1. The biological process has been effective in the reduction of the ampicillin and chloramphenicol-resistant total coliforms and *E. coli* by about 2-log units; 2. Ampicillin-resistant *E. coli* colonies increase compared to ampicillin-resistant coliforms with increasing ampicillin concentration; 3. Ampicillin and ampicillin/chloramphenicol-resistant *E. coli* colonies were found to be related to the presence of the *tem1* gene.

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Wen et al. (2016)	1. Study of the distribution and removal efficiency of *intI1* and eight subtypes of ARGs for tetracycline, sulfonamides and beta-lactam resistance in four municipal wastewater treatment plants.	WWTP1: A²/O process, WWTP2: A/O process WWTP3: CAS system WWTP4: Cyclic CAS system (CASS).	Beijing, China.	-	-	**ARGs:** *tetA, tetO, tetW, sul1, sul2, blaCTX-M, blaTEM, blaSHV, IntI1.*		Conventional PCR, qPCR after cloning.	1. *IntI1* and the 6 ARGs tested except *blaTEM, blaSHV* were detected in WW and sludge samples; 2. The removal efficiency of different ARGs were 0.3-2.7 orders of magnitude in the four WWTPs; 3. It was shown that biological treatment plays the most important role in ARGs removal (1.2-1.8 orders of magnitude of removal efficiency) of all treatment steps followed by UV disinfection process; 4. Although all WWTPs with different treatments can reduce ARGs content effectively, ARGs concentrations are still high in the effluent.
Li et al. (2016)	1. Investigation of the occurrence and removal of targeted pollutants using different processes in two WWTPs and in the downstream water.	WWTP-Q: A²/O process WWTP-L: triple oxidation process (OX).	Eastern region, China.	Total heterotrophic bacteria resistant to tetracycline, sulfamethoxazole and to the mixture of the two.	**Antibiotics:** Three tetracyclines, four sulfonamides, trimethoprim.	**ARGs:** *tet(A), tet(B), tet(C), tet(G), tet(L), tet(M), tet(O), tet(Q), tet(W), tet(X), sul1, sul2, and intI1*	Total DNA.	Isolates from non-selective medium, qPCR.	1. Biological treatment mainly removed antibiotics and ARGs, whereas physical techniques were found to eliminate ARB; 2. UV disinfection did not significantly enhance the removal efficiency, so the release of the available target contaminants from the excess sludge may pose threats to human and the environment; 3. Different antibiotics showed diverse influences on the downstream lake, and the concentrations of sulfamethazine and SMX were observed to increase enormously; 4. High concentrations of antibiotics and abundance of ARGs and ARB were found in the excess sludge samples.

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\multicolumn{10}{c}{**FATE OF ARB&ARG DURING BIOLOGICAL WASTEWATER TREATMENT**}
Tao et al. (2016)	1. Explore the abundance and diversity of ARGs and MGE in WWTP sludge; 2. Reveal the potential differences of ARGs between anaerobic and aerobic treatment processes; 3. Investigation of the potential reasons for the variation of ARGs abundances among different treatment processes.	Pharmaceutical WWTP A: two hydrolytic acidification systems (HA), a CASS and an A/O system. Pharmaceutical WWTP B: Up-flow blanket filter system (UBF) and A/O system. Urban WWTP1 and 2: A^2/O system. Urban WWTP3: oxidation ditch.	Hangzhou City (WWTPA),Taizhou (WWTPB) and the remain three in Zhengzhou, China		-	Metagenomic examination; **ARGs:** 215 ARG subtypes belonging to 11 ARG types (aminoglycosides, beta-lactams,chloramphenicol, fosfomycin, macrolide-lincosamide-streptogramin (M-L-S), multidrug, polypeptides, quinolones, sulfonamides, tetracyclines and unclassified); MGE.	Total DNA	Illumina Hiseq sequencing, metagenomic sequencing, searching using BLASTn against databases (Antibiotic Resistance Genes Database, the Integron Database, Insertion Sequences Database and NCBI Reference Sequence Database).	1. The results showed that ARGs abundances in WWTPA and B sludge were higher than those in STP sludge; 2. The diversity of ARGs in WWTP aerobic sludge (153 subtypes) was higher than that in STP aerobic sludge (118 subtypes); 3. The profiles of ARGs in PWWTP aerobic sludge were similar to those in STP aerobic sludge but different from those in PWWTP anaerobic sludge, suggesting that dissolved oxygen (DO) could be one of the important factors affecting the profiles of ARGs; 4. In PWWTP aerobic sludge, aminoglycoside, sulfonamide and multidrug resistance genes were frequently detected. 5. Tetracycline,macrolide-lincosamide-streptogramin and polypeptide resistance genes were abundantly present in PWWTP anaerobic sludge; 6. Significant correlations between ARGs types and seven bacterial genera were found. In addition, the MGE were also examined and correlations between the ARGs and MGE in PWWTP sludge were observed.
Mao et al. (2015)	1. Determination of the concentrations and flow of 23 of the most frequently detected ARGs through each WWTP treatment unit,	Anaerobic and anoxic lagoons followed by a CAS process.	Northern region, China.	Total heterotrophs.	**Antibiotics:** Antibiotics belonging to the tetracycline, sulfonamide, quinolone, macrolide families. **Heavy metals:** (i.e., As,	**ARGs:** tetA, tetB, tetC, tetD, tetE, tetG, tetH, tetJ, tetK, tetL, tetM, tetO, tetQ, tetT, tetW, tetX, tetZ, tetA/P,	16S rRNA	Isolates from selective medium, qPCR.	1. Bacteria harbouring ARGs persisted through all treatment units, surviving disinfection by chlorination in greater percentages than total bacteria; 2. The absolute abundances of ARGs were reduced from the raw influent to the effluent by 89.0% -99.8%,

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	treated WWTP effluent, and discharged biosolids; 2. Quantification of how individual wastewater treatment processes affect the fluxes of antimicrobial drugs, antimicrobial resistant organisms, and antimicrobial resistance elements (e.g., their associated DNA).				Cd, Cr, Cu, Ni, Pb and Zn) in sewage and activated sludge samples.	*tetB/P, sul1, sul2, sul3, sulA, qnrA, qnrB, qnrD, qnrS, ermB, ermC,* 16S rRNA.			although considerable ARGs levels were found in WWTP effluent samples; 3. Twelve ARGs (*tetA, tetB, tetE, tetG, tetH, tetS, tetT, tetX, sul1, sul2, qnrB, ermC*) were discharged through the dewatered sludge and plant effluent at higher rates than influent values, indicating overall proliferation of resistant bacteria; 4. The correlation between the concentrations of various heavy metals and the enrichment of some ARGs may alleviate their selective pressure and mitigate ARGs proliferation in WWTPs.
Al-Jassim et al. (2015)	1. Assessment of the removal efficiency of microbial contaminants in a local WWTP over the duration of one year 2. Assessment of the microbial risk associated with reusing treated wastewater in agricultural irrigation.	Initial settling of solids in a primary clarifier, followed by CAS treatment.	Jeddah, Saudi Arabia.	Ampicillin, kanamycin, erythromycin, tetracycline, ceftazidime, ciprofloxacin, chloramphenicol and meropenem-resistant heterotrophic bacteria and coliforms.	**Physicochemical parameters:** Suspended solids (TSS) and chlorine.	**ARGs:** *tetO, tetQ, tetW, tetH, tetZ,* 16S rRNA.	Total DNA	Isolates from non-selective medium, ion PGM, qPCR.	1. The treatment process achieved 3.5 logs removal of heterotrophic bacteria and up to 3.5 logs removal of fecal coliforms; 2. 16S rRNA gene-based high-throughput sequencing showed that several genera associated with opportunistic pathogens (e.g. *Acinetobacter, Aeromonas, Arcobacter, Legionella, Mycobacterium, Neisseria, Pseudomonas* and *Streptococcus*), were detected at relative abundance in the influent and some of them were found in chlorinated effluent and non-chlorinated effluent; 3. The proportion of bacterial isolates resistant to 6 types of antibiotics increased from 3.8% in the influent to 6.9% in the chlorinated effluent;

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									4. *tetO, tetQ, tetW, tetH, tetZ* were also present in the chlorinated effluent.
Christgen et al. (2015)	1. Use of a metagenomic approach to contrast the fate of ARGs in anaerobic, aerobic, and AAS bioreactors treating domestic wastewater.	Upflow anaerobic sludge blanket reactor (UASB), anaerobic hybrid reactor (AHR), and a completely mixed aerobic reactor. Effluents from the AHR and UASB units were pumped into second-stage aerobic units (AER2 and AER3) for further treatment, respectively (the two anaerobic−aerobic sequence (AAS) systems were AHR−AER2 and UASB−AER3).	Northern England.	Metagenomic examination.	-	**ARGs:** 230 ARG.	Total DNA.	Illumina HiSeq Sequencing, Metagenome sequences searched using BLASTx against the clean Antibiotic Resistance Database (ARDB).	1. The AAS and aerobic treatment achieved a higher removal of certain ARGs (aminoglycoside, tetracycline, betalactam resistance genes) compared to UASB and AHR, indicating the higher capacity of the combined system to remove ARGs compared to each process alone; 2. Sulfonamide and chloramphenicol resistance genes were unaffected by the AAS treatment while the phenotypic prevalence of ARGs shifted from target-specific ARG to multi-drug resistance, from influent to effluent; 3. Metagenomic data suggested that aerobic processes may be generally better than anaerobic processes for reducing ARGs sequences through treatment and AAS can provide equivalent treatment performance and ARGs reduction with less energy(~32% in this study).
Du et al. (2015)	1. Investigation of the variation of ARGs in wastewater in sludge throughout the treatment process, in order to provide a scientific basis for controlling ARGs in WWTPs.	A²/O-MBR process.	Jiangsu province, China.	-	**Physicochemical parameters:** COD, TP, TN, TOC, NH.	**ARGs:** *tetG, tetW, tetX, sul1, intI1.*	genomic DNA.	qPCR.	1. ARGs concentrations reduced in the influent and effluent samples as: *sul1>intI1>tetX>tetG>tetW;* 2. All ARGs concentrations were higher in spring compared to other seasons; 3. ARGs concentrations decreased in the anaerobic and anoxic effluent but increased in the aerobic effluent and sharply declined in MBR effluent; 4. The reduction in *tetW, intI1* and *sul1* were positively correlated to 16S rDNA concentration.

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Yang et al. (2014)	1. Understanding the occurrence and abundance of ARGs; 2. Identification of the persistent ARGs in a full-scale WWTP; 3. Further determination of the fate of ARGs through sewage/sludge treatment; 4. Determination of the impacts of microbial community composition on ARGs.	CAS system.	Hong Kong, China.	Metagenomic examination.	-	**ARGs:** 271 ARG.	Total DNA.	Illumina Hiseq sequencing, metagenome sequences searched using BLASTx against the clean Antibiotic Resistance Database (ARDB).	1. 271 ARGs subtypes belonging to 18 ARG types were identified by the broad scanning of metagenomic analysis; 2. Influent had the highest ARGs abundance, followed by effluent, anaerobic digestion sludge and activated sludge; 3. 78 ARGs subtypes persisted through the biological wastewater and sludge treatment process; 4. The high removal efficiency of 99.82% for total ARGs in wastewater suggested that sewage treatment process is effective in reducing ARGs; 5. The removal efficiency of ARGs in sludge treatment was not as good as that in sewage treatment; 6. Significant correlation between 6 genera and the distribution of ARGs were found and 5 of the 6 genera included potential pathogens.

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Novo et al. (2013)	1. Correlation of the occurrence of antimicrobial residues and ARB in sewage with the structure and composition of the bacterial community and the antibiotic resistance loads of the final effluent.	Primary settling tank followed by a CAS biological process and a secondary settling tank to remove the biomass and other suspended particles.	Northern region, Portugal.	Bacterial community, total and amoxicillin, tetracycline , ciprofloxacin and sulfamethoxazole-resistant total heterotrophic bacteria, enterobacteria and enterococci.	**Antibiotics:** Oxytetracycline,doxycycline tetracycline, chlortetracycline, penicillin G, penicillin V, sulfathiazole, sulfamethazine,enrofloxacin, ciprofloxacin, ofloxacin and triclosan **Metals:** Cadmium, lead,chromium arsenic and mercury. **Physicochemical parameters:** temperature, COD, BOD, water flow.			Isolation in non-selective medium, 16S rRNA DGGE.	1. The bacterial community structure was distinct in raw and in treated wastewater and varied over time; 2. Temperature and COD were correlated with the variation of the bacterial community structure of raw and treated wastewater, respectively; 3. Regarding amoxicillin, tetracycline and sulfonamide resistance, the antibiotic resistance percentages differed between Autumn and Spring periods; 4. In Autumn, but not in Spring, amoxicillin and ciprofloxacin resistance prevalence increased significantly after wastewater treatment; 5. Temperature was positively correlated with the prevalence of sulfonamide resistant heterotrophs and enterobacteria in treated wastewater; 6. Tetracycline concentration in the raw wastewater was positively correlated with the antibiotic resistance prevalence in treated wastewater, although not specifically with tetracycline resistance; 7. The concentration of tetracyclines, penicillins, sulfamides and quinolones and the abundance of antibiotic-resistant cultivable bacteria in the raw wastewater were positively correlated with the abundance of *Epsilonproteobacteria* in treated wastewater and negatively with *Gamma, Betaproteobacteria* and *Firmicutes*.

Refere-nce	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experime-ntal methods	Study findings
FATE OF ARB&ARG DURING BIOLOGICAL WASTEWATER TREATMENT									
Su et al. (2013)	1. Investigation of the antibiotic resistance profiles in *E. coli* isolated from 2 WWTP; 2. Detection of plasmid-mediated quinolone resistance genes, *ampC* gene and integrons by PCR; 3. Assessment of the effects of treatment processes on the removal of ARB&ARG in WWTP.	A: pre-treatment, grit chamber and CCAS system including A²/O process, secondary clarifier and chlorination. B: pre-treatment, grit chamber, oxidation ditch and secondary clarifier followed by UV-C disinfection.	Guangdong province, China	ampicillin, piperacillin, cefalozin, ceftazidime, gentamycin, streptomycin, ciprofloxacin, levofloxacin, sulfamethoxazole/trimethoprim, tetracycline, chloramphenicol-resistant *E. coli*	-	*qnrA, qnrB, qnrS, aac(6')-lb-cr, ampC, intI1, intI2, intI3*	genomic DNA	Membrane filtration method using modified mTEC agar followed by the Kirby-Bauer disk diffusion test, conventional PCR.	1. 98.4% of the isolates were resistant to the examined antibiotics. 90.6% were resistant to at least 3 antibiotics; 2. Tetracycline had the highest resistance frequency followed by ampicillin; 3. *IntI1* had the highest detection rate in the plasmids; 4. The disinfection process could significantly reduce total bacteria but not ARB, as resistance frequency to each antibiotic and plasmid-mediated resistance genes detection rate increased in the effluent of the biological units.

Despite the fact that the conventional activated sludge process, which is widely used for wastewater treatment makes a fascinating research epicentre due to its highly diverse microorganism communities and richness of content, the majority of studies deal with the fate of ARB&ARGs during wastewater treatment in the context of the whole WWTP and not solely with the conventional activated sludge (CAS) treatment as such. As a result, the results provided by the studies given, include ARB&ARGs concentrations found in the final effluents of the investigated WWTP, which may include treatment steps beyond the biological process step, such as a disinfection step (i.e. chlorination, UV-radiation).

2.1.1. *There is variable efficiency during WWTP processes for the efficient reduction of ARB&ARGs*

The fate of ARB&ARGs was investigated in various studies, in order to determine the efficiency of the applied treatment and establish the best conventional treatment processes. It has been observed in studies conducted in the last 5 years, that despite the fact that absolute abundances of ARB&ARGs decrease (number of gene copies per ng of DNA or per volume of water sample), there is an increase in relative abundance of ARGs (number of gene copies per 16S rRNA copy) found in treated effluents.

More specifically, the highest counts of beta-lactam, tetracycline and fluoroquinolone-resistant *E. coli* were found in untreated wastewater in a study in Poland (Osińska et al., 2017), while Su et al. (2014) found that the most prevalent resistance type was the one of tetracycline, which had the highest frequency among examined isolates followed by ampicillin resistance. Zanotto et al. (2016) demonstrated the reduction of ampicillin- and chloramphenicol-resistant *E. coli* by 2-logs in a peracetic acid-treated effluent. However, chlorination and UV-C disinfection processes also examined in this study after biological treatment were shown to effectively reduce the concentrations of total bacteria but not of ARB, as the prevalence of ARB after disinfection and plasmid-mediated resistance gene detection rate seemed to increase. Su et al. (2014) examined the efficiency of two biological treatment processes for the reduction of antibiotic-resistant *E. coli* (cyclic CAS system followed by chlorination and oxidation ditches followed by UV-C disinfection). These authors found, in agreement with the abovementioned studies, that 98.4% of the isolates were resistant to at least one of 12 examined antibiotics, while 90.6% of the total examined isolates were resistant to at least 3 of the 12 antibiotics, thus conferring multi-drug resistance (MDR). A high

proportion of multi-drug resistant *E. coli* isolates (38%) was identified in a study by Osińka et al. (2017) in untreated and treated effluents, while 43% of these were found in untreated WW. In a different study, Al-Jassim et al. (2015) investigated the fate of multi-drug resistant bacteria to 6 types of antibiotics, during the CAS process followed by chlorination. The results showed that there was a slight increase in the proportion of the bacterial isolates that were resistant to all 6 types of antibiotics among all isolates, from the influent to chlorinated effluent, from 3.8% to 6.9%.

Vancomycin-resistant enterococci (VRE) prevalence was examined in another study by Goldstein et al. (2014), in a WWTP influent and effluent. It was shown that VRE were present in influent and biologically treated samples (activated sludge, post-aeration and secondary clarifiers), but they were absent in the tertiary-treated effluent by chlorination, a fact that indicates the efficiency of chlorination in the effective reduction of genetic components of the biologically-treated wastewater. In the same study however, a sequencing batch reactor consisting of 5 lagoons was able to effectively remove VRE and methicillin-resistant *Staphylococcus aureus* (MRSA) without the need of disinfection or filtration. Ozone treatment as a tertiary treatment following biological treatment was less effective in the reduction of ARGs than UV-C and chlorination (Ben et al. 2017).

UV-C tertiary treatment following biological CAS treatment was not shown to enhance the reduction of ARGs in a study by Li et al. (2015) compared to the secondary biological process applied, releasing equivalent ARGs concentrations to those released by the secondary CAS treatment, in the treated effluents. This is in agreement with Mao et al. (2015), where considerable relative ARGs concentrations were shown to be found in chlorinated effluent, despite the fact that there was a 89%-99.8% decrease in ARGs from influent to chlorinated effluent. This statement is supported by another study where it was shown by Mao et al. (2015) that bacteria harbouring ARGs were able to persist throughout tertiary chlorination treatment, increasing in prevalence compared to the total bacterial population, suggesting the overall proliferation of ARB during the WWTP processes.

A study by Rafraf et al. (2016) showed that despite the fact that the relative concentration of ARGs to the total amount of genomic DNA decreased after treatment compared to the untreated WW, the absolute abundance of the ARG *blaCTX-M*, *blaTEM* and *qnrS* was higher in the effluent of the WWTP, indicating a tolerance of the specific ARGs to secondary and tertiary treatment. Nevertheless, in another study, the

absolute abundance of ARGs was shown to decrease in the effluent compared to the influent, in a study by Yang et al. (2014) by 99.82%, where 271 ARGs subtypes were identified, belonging to 18 ARGs types. A similar finding was observed by Du et al. (2015), where the concentration of 16S rRNA was statistically correlated to the concentration of ARGs in an A^2/O treated effluent, indicating that as the total 16S rRNA amount decreased, there was a respective ARGs reduction. However, in the study by Yang et al. (2014), the anaerobically digested sludge and the activated sludge used during the CAS biological treatment were found to have a lower absolute abundance of ARGs compared to influent and effluent samples. It is noteworthy that this study also identified 78 subtypes of ARGs that were persistent during biological (CAS) treatment of wastewater even through the sludge treatment process, indicating the inability of biological processes, and their subsequent sludge treatment processes, to effectively reduce ARGs concentrations and types, allowing their potential transfer into the environment and subsequent proliferation. In another study by Bengtsson-Palme et al. (2016), it was shown that WWTP process could decrease the absolute abundance of human associated total and ARB. However, the relative abundance and diversity of ARGs was found to be less affected, i.e. in the case of mobile genetic elements such as the class 1 integron (*IntI1*), which were not significantly reduced in the effluent water compared to the effluent.

2.1.2. *Specific bacterial genera are suggested to be involved in the transfer and proliferation of ARGs during biological treatment processes*

According to Tao et al. (2016), there was a significant statistical correlation between the distribution of ARGs and several bacterial genera, suggesting the involvement of the identified bacterial genera in the transfer and proliferation of the examined ARGs. 16S rRNA gene-based high-throughput sequencing of influent and chlorinated effluent samples by Al-Jassim et al. (2015) showed the presence of several genera associated with opportunistic pathogens, such as *Acinetobacter, Aeromonas, Arcobacter, Legionella, Mycobacterium, Neisseria, Pseudomonas* and *Streptococcus*. Besides, in a study by Yang et al. (2014), there was significant statistical correlation between 6 identified bacterial genera and the overall distribution of the ARGs during the different steps of the wastewater treatment, with *Proteobacteria* found in all treatment steps and *Firmicutes,* a genera that is highly prevalent in anaerobic conditions, being the most dominant genera in influent and activated digested sludge samples. *Actinobacteria* and

Bacteroidetes were more prevalent in treated effluent samples, indicating a change in the bacterial community structure during wastewater treatment, leading to shifts in the dominant microorganism genera in each community. This is in agreement with Novo et al. (2013), whose study showed a distinct bacterial structure in raw and treated wastewater samples, which varied over time, during treatment. The structure of the bacterial community in treated wastewater was also affected by the concentration of antibiotic residues in the examined samples. More specifically, the concentrations of tetracyclines, penicillins, sulfonamides and quinolones and the abundance of antibiotic resistant cultivable bacteria in the raw wastewater were positively correlated with the abundance of *Epsilonproteobacteria* in treated wastewater and negatively with *Gamma*, *Betaproteobacteria* and *Firmicutes*. In a study by Zhang et al. (2015), 16S rRNA gene sequence analysis showed 18 genera among 188 isolates in WWTP influent and effluent samples. Only 6 genera (*Aeromonas, Bacillus, Lysinibacillus, Microbacterium, Providencia* and *Staphylococcus*) were found in the examined influent and effluent samples. However, at the same time, abundance of 13 ARGs increased in the aerobic compartments of the WWTP compared to the anoxic tank ARGs concentrations, highlighting the effect of aerobic environments to the dissemination of ARGs.

The microbial community found in a WWTP undigested surplus sludge was examined and compared to the treated effluent community, in a study by Bengtsson-Palme et al. (2016). It was shown that the microbial genera were similar in the surplus sludge to those found in the wastewater effluent. However, in the sludge there were bacterial phyla that were not observed in the effluent, such as *Acidobacteria* and *Nitrospirae*. The digested sludge however demonstrated a clear shift in the present community, with a great increase in the prevalence of *Cloroflexi* and *Spirochaetes* phyla, which include many anaerobes, as well as higher abundances of Archaea. In these samples the *Proteobacteria* were greatly decreased while there were no Eukaryota found. It should be noted that the same study noted a major reduction in the relative abundance of human-associated bacteria from the influent to the effluent, which was driven by *Acinetobacter* and *Streptococcus*, which constituted 13.6% of the identified bacteria in the influent. Legionella was not efficiently removed by the WWTP process, and was found to be enriched in the effluent, highlighting its potential significance as an antibiotic resistance disseminator.

2.2. Impacts of biological treatment processes on the receiving environments

There are considerable risks to be taken into account regarding discharge of WWTP-treated effluents into aquatic receiving bodies, such as rivers and lakes. Treated effluents may be potential pollutant insertion sources via deposition mechanisms into sediments, accumulation of discharged chemical and biological/microbiological microcontaminants contained in these effluents (Table 2).

Table 2 - Comprehensive review of the studies examining the impacts on the receiving environment of biological wastewater treatment processes.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
	**IMPACTS ON THE RECEIVING ENVIRONMENT OF BIOLOGICAL WASTEWATER TREATMENT PROCESSES**								
Lopes et al. (2016)	1. To assess the occurrence of thermotolerant coliforms and *E. coli* isolates resistant to antimicrobials in an WWTP effluent; 2. Assessment of the risks related to the insertion of resistant microorganisms in the local ecosystem. 3. Examination of the relation of the presence of these microorganisms with physical and chemical parameters.	Anaerobic fluidized bed reactor (RALF).	Medianeira, west Parana state, Brazil.	norfloxacin, ciprofloxacin, cephalothin, gentamycin, streptomycin, imipenem, cefaclor, ampicillin, cefoxitin, tetracycline, amoxicillin, chloramphenicol-resistant thermotolerant coliforms and *E. coli*.	**Physicochemical parameters:** pH, oxygen saturation, total solids, fixed total solids, volatile total solids, temperature.	-	-	Antimicrobial susceptibility method using isolates from non-selective medium.	1. There were *E.coli* isolates resistant to cephalothin, streptomycin, tetracycline and amoxicillin; 2. A higher prevalence of resistant isolates was observed in the WWTP effluent and downstream of the WWTP.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
					IMPACTS ON THE RECEIVING ENVIRONMENT OF BIOLOGICAL WASTEWATER TREATMENT PROCESSES				
Li et al. (2016)	1. Occurrence and removal of targeted pollutants using different processes from two WWTPs and in the downstream river (lake basin).	WWTP-Q treats domestic and industrial sewage through primary clarification A^2/O process followed by UV disinfection. WWTP-L uses triple oxidation process (OX) followed by UV disinfection. The receiving environment is a lake spreading across 10 km^2 with an average water stage of 25 m throughout the year.	Zhejiang province, China.	Heterotrophic plate counts resistant to tetracycline, sulfamethoxazole, mixture of the two	**Antibiotics:** sulfamethazine, sulfamethoxazole, thiabendazole, trimethoprim, oxytetracycline, chlortetracycline tetracycline	**ARGs**: *tetA, tetB, tetC, tetG, tetL, tetM, tetO, tetQ, tetW, tetX, sul1, sul2, intl1*	16S rRNA.	Isolates from non-selective medium, qPCR.	1. The biological treatment was most efficient on the removal of antibiotics and ARGs; 2. Physical techniques were more efficient in the elimination of ARB abundance (1 log reduction); 3. UV disinfection did not significantly contribute to the removal efficiency; 4. Different antibiotics showed diverse influence on the downstream lake. The concentrations of sulfamethazine and sulfamethoxazole increased significantly in the lake; 5. Total ARGs abundance increased by 0.1 log and some ARGs such as *tetC*, *intl1* and *tetA* increased, possibly due to the high input of the efluent.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
**IMPACTS ON THE RECEIVING ENVIRONMENT OF BIOLOGICAL WASTEWATER TREATMENT PROCESSES**									
Proia et al. (2016)	1. Study of the effect of WWTP effluents on the prevalence of ARGs in river biofilms.	-	Tordera river basin, Catalonia, Spain.	Tetracycline and sulfamethoxazole-resistant heterotrophic plate counts	**Physicochemical parameters:** Ash free dry mass, pigment extraction for chl-α determination, autotrophic index, Conductivity, temperature, pH, DO, TOC, TP, TN.	**ARGs:** *blaCTX-M, qnrS, sul1, ermB.*	16S rRNA.	qPCR.	1. WWTP effluents significantly altetred the hydrology, physico-chemistry and biological characteristics of the receiving streams; 2. The WWTP effluents also favoured the persistence and spread of ARGs in microbial benthic communities; 3. The magnitude of the effect depends on the relative contribution of each WWTP to the receiving system; 4. Low ARGs abundance was observed upstream of the WWTPs compared to the abundance downstream of WWTP discharge points. 5. ARGs abundance was noticeable even 1 km downstream of WWTP discharge.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
					IMPACTS ON THE RECEIVING ENVIRONMENT OF BIOLOGICAL WASTEWATER TREATMENT PROCESSES				
Osińska et al. (2016)	1. Determination of the number of fluoroquinolone-resistant bacteria and their share in total heterotrophic plate counts in treated wastewater, upstream and downstream river water samples where TWW is discharged; 2. Determination of the presence/absence of resistance genes to the most popular antibiotic groups (tetracyclines and beta-lactams).	Mechanical, biological, and chemical treatment sections and sludge processing.	Olsztyn, Poland.	Fluoroquinolone-resistant bacteria, to amoxicillin/clavulanic acid, mezlocillin, piperacillin, ceftazidime, cefotaxime, tigecycline, tobramycin, trimethoprim/sulfamethoxazole, tetracycline	-	**ARGs:** *aac(6')-Ib-cr, qnrA, qnrB, qnrD, qnrS, qepA, oqxA, oqxB, blaCTX-M, blaCTX-M-1, blaCTX-M-2, blaCTX-M-9, blaSHV, blaTEM, blaOXA, tetA, tetB, tetC, tetD, tetE, tetG, tetK, tetL, tetM, tetO, tetS, tetQ, tetX, tetP*	Total DNA.	Isolates from selective medium, Conventional PCR	1. The prevalence of fluoroquinolone-resistant bacteria was 0.7% in upstream river water and 7.5% in treated WW effluent; 2. *Escherichia* (25%), *Acinetobacter* (25%) and *Aeromonas* (6.9%) genera were the most predominant fluoroquinolone-resistant genera; 3. Fluoroquinolone resistance was mostly caused by the presence of the gene *aac(6')-1b-cr* (91.4 %); 4. The most prevalent ARG associated to beta-lactam resistance were *blaTEM, blaOXA* and *blaCTX-M*; 5. The highest prevalence of multidrug-resistant (MDR) microorganisms was detected in TWW and downstream river water samples.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
IMPACTS ON THE RECEIVING ENVIRONMENT OF BIOLOGICAL WASTEWATER TREATMENT PROCESSES									
Tang et al. (2016)	1. Investigate the changes of bacterial community and ARGs along treatment processes of one WWTP; 2. examine the effects of the effluent discharge on the bacterial community and ARGs in the receiving river.	Activated sludge and integrated A²/O treatment process.	Beijing, China.	Metagenomic analysis	-	23,137 sequences grouped into 380 ARGs groups encoding resistance to 249 antibiotics from the Antibiotic Resistance Database (ARDB)	Total DNA.	454 pyrosequencing, Illumina Hiseq sequencing.	1. High removal of ARGs examined by the UWTP process; 2. No significant differences were found in the bacterial community downstream and upstream the river although potential pathogens and ARGs were slightly higher abundance in downstream water samples.
Rowe et al. (2016)	1. Describe the ARGs content of two distinct effluents that enter a single river catchment; 2. Characterisation of the MGE and pathogenic bacteria present; 3. Relate the abundance of these features to a background sample of the river source water, taken from upstream of the effluent entry points.	-	Cambridge, UK.	Metagenomic analysis.	-	**ARGs:** ARGs and mobile genetic elements (MGEs).	Total DNA.	Illumina HiSeq sequencing, search Engine for Antimicrobial Resistance (SEAR) pipeline for ARGs and Burrows-Wheeler Alignerment for MGE.	1. Effluents were found to contain an array of ARGs, MGE and pathogenic bacteria more diverse and abundant compared to a background sample of the river. 2. Most abundant ARGs found were tetracycline resistance genes *tetC* and *tetW* from farm effluents and the sulfonamide resistance gene *sul2* from WWTP effluents. 3. Compared to the background sample taken up river from effluent entry, the average abundance of genes was three times greater in the farm effluent and two times greater in the WWTP effluent.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
IMPACTS ON THE RECEIVING ENVIRONMENT OF BIOLOGICAL WASTEWATER TREATMENT PROCESSES									
Taučer-Kapteijn et al. (2016)	1. Investigation of the presence of ampicillin and vancomycin-resistant enterococci in the treated effluent of six wastewater treatment plants (WWTPs) and in surface water used as a source for drinking water production in the Netherlands.	Bar screens, grit chambers, primary sedimentation, aeration, activated sludge and a second sedimentation step.	Western Netherlands.	Ampicillin-resistant *Enterococcus* (ARE), vancomycin-resistant *Enterococcus* (VRE) and total enterococci.	**Antibiotics:** three tetracyclines, four sulfonamides, trimethoprim.	**ARGs:** *vanA and vanB*	Total DNA.	MALDI-TOF MS, Conventional PCR, Vitek 2 system.	1. ARE was observed in all studied UWTP effluents while VRE was not detected; 2. No VRE or ARE were detected in surface water samples; 3. Intrinsic vancomycin-resistant *Pediococcus* spp., *Leuconostoc* spp. and *Lactobacillus* spp. were isolated with the vancomycin medium; 4. The ARE isolates did not contain the *vanA* or *vanB* gene, but MIC testing for vancomycin showed intermediate vancomycin resistance (2–8 g mL^{-1}) to occur in these strains. 5. The discharge of ampicillin resistant E. faecium strains with intermediate vancomycin resistance by the WWTPs into the surface water was found, but no presence of these strains downstream at intake locations for drinking water production.
Berglund (2015)	1. Assessing the impact of WWTP effluent on relative abundance of ARGs and integrons in the receiving river.	-	Linköping, Sweden.	-	**Antibiotics:** Ciprofloxacin, clarithromycin and clindamycin	**ARGs:** sul1, dfr1, ermB, tetA, tetB, vanB, qnrS, intI1	16S rRNA.	qPCR.	1. An increase in examined ARGs and integrons downstream of the WWTP was observed. 2. Low concentration of examined antibiotics was found in the river samples. 3. The downstream increase in ARGs prevalence is likely to be attributable to accumulation of genes present in the treated effluent discharged from the WWTP sludge were observed.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
IMPACTS ON THE RECEIVING ENVIRONMENT OF BIOLOGICAL WASTEWATER TREATMENT PROCESSES									
Xu et al. (2015)	1. Determination of the abundance and distribution of antibiotics and ARGs from a sewage treatment plant and its effluent-receiving river in Beijing, China.	Aerobic/anoxic biological treatment process.	Beijing, China.		**Antibiotics:** Oxytetracycline, tetracycline, chlortetracycline, sulfacetamide, sulfathiazole, sulfapyridine, sulfamerazine, sulfadimidine, sulfachloropyridazine, sulfamethoxazole, sulfadimethoxine, sulfamethoxypyridazine, sulfachinoxaline, ofloxacin, enrofloxacin, ciprofloxacin and norfloxacin.	**ARGs:** *tetA, tetB, tetC, tetE, tetW, tetM, tetT, tetZ, tetB, sul1, sul2, sul3, sulA, gryA, qnrB, qnrC, qnrD, parC, qnrS.*	Genomic DNA.	qPCR.	1. Sulfonamide resistance genes were present at relatively high concentrations in all samples; 2. All ARGs abundances remained stable along each step of WWTP process; 3. The abundances of detected ARGs from the WWTP were also higher than its receiving river; 4. Correlation analyses showed that *tetB* and *tetW* abundances were strongly correlated to tetracycline residual concentrations; 5. The WWTP process may induce the increase of prevalence of the examined ARGs.
Kotlarska et al. (2015)	1. Investigatation of antibiotic resistance profiles in *E. coli* isolated from two local WWTP (raw and treated wastewater samples), their marine outfalls located in the Gulf of Gdansk, the Baltic Sea (Poland), and from major tributary of the Baltic Sea, the Vistula River.	Integrated effective removal of nitrogen, phosphorus, and carbon in anaerobic/anoxic/oxic zones fed with internal recycles (Gdansk–Wschod WWTP) and four-stage system at the biological stage of the plant, including primary and secondary anoxic reactors (Gdynia–Debogorze).	Gulf of Gdansk, Poland.	Amikacin, gentamicin, tobramycin, imipenem, meropenem, cefazolin, cefuroxime, ceftazidime, cefotaxime, cefepime, aztreonam, ampicillin, amoxicillin/clavulanate, piperacillin/tazobactam, trimethoprim/sulfamethoxazole, ciprofloxacin, and	-	**ARGs:** *intI1, intI2, sul1 and qacEΔ1.*	Total DNA.	Isolates from no selective medium, conventional PCR, amplicon sequencing.	1. Ampicillin-resistant *E. coli* were most frequent, followed by amoxicillin/clavulanate, trimethoprim/sulfamethoxazole and fluoroquinolone-resistant isolates; 2. 32.1% and 3.05% of *E. coli* isolates were positive for the presence of class 1 and 2 integrons, respectively; 3. The presence of integrons was associated with increased frequency of resistance to fluoroquinolones, trimethoprim/sulfamethoxazole, amoxicillin/clavulanate, piperacillin/tazobactam and multidrug-resistance phenotypes;

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
									IMPACTS ON THE RECEIVING ENVIRONMENT OF BIOLOGICAL WASTEWATER TREATMENT PROCESSES
				levofloxacin together with screening for extended-spectrum beta lactamase-resistant *E. coli*. Metagenomic analysis.					4. Nine gene cassette arrays were confirmed among sequenced variable regions with predominance of d*frA1-aadA1, dfrA17-aadA5* and *aadA1* array.
Naquin et al. (2015)	1. The evaluation of the presence of ARG in a small rural WWTP.	Primary and secondary CAS treatment followed by tertiary UV treatment.	Thibodaux, Louisiana, USA.	Erythromycin, tetracycline, methicillin-resistant total heterotrophs.	**Physicochemical parameters:** pH, COD.	**ARGs:** *ermB, sul1, tetA, tetW, tetX, mecA*	16S rRNA.	Plate-spread method, qPCR.	1. ARG were present in both raw and treated sewage. 2. There was evidence of successful transformation of methicillin resistance gene, *mecA* to *Staphylococcus aureus* within 24 hours.
Marti et al. (2014)	1. Characterisation of ciprofloxacin-resistant isolates from a WWTP discharge point and its receiving river; 2. Examination of the prevalence of PMQR determinants in these isolates; 3. Determination of harbouring of plasmid-encoded β-lactamases such as TEM, SHV and CTX-M.	-	Barcelona, Spain.	Amoxicillin, ampicillin, penicillin, carbenicillin, cefalotin, nalidixic acid, norfloxacin, pipemidic acid-resistant *E. coli*. Metagenomic analysis.	Erythromycin, azythromycin, clarithromycin, spyramycin, cefotaxime, pipemidic acid, ciprofloxacin, difloxacin, enrofloxacin, flumequine, ofloxacin, norfloxacin, sulfamethoxazole, sulfathiazole, sulfapyridine, chlortetracycline, clindamycin, trimethoprim.	Phylogenetic identification of isolates **ARGs:** *qnrA, qnrB, qnrS, aac(60)-Ib-cr*, qnr-positive plasmids from isolates	16S rRNA	Conventional PCR, Amplicon sequencing, multiplex PCR, disk diffusion susceptibility testing, UPLC-MS.	1. Antibiotics were found in the range of ng L⁻¹ in WWTP effluents, but most of them were no longer found in downstream river. 2. Some fluoroquinolones were detected in sediment downstream demonstrating their high persistence and their capacity to be retained in the river sediments. 3. Most of the ciprofloxacin-resistant isolates belonged to the *Gammaproteobacteria* class and 17 of them, 8 (7.6%) from the first sampling and 9 (6.1%) from the second sampling,

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experime ntal methods	Study findings
IMPACTS ON THE RECEIVING ENVIRONMENT OF BIOLOGICAL WASTEWATER TREATMENT PROCESSES									
									carried a *qnr* gene. 4. Among the *qnr*-positive isolates, 12 harboured the *aac(6')-lb-cr* gene and 2 of them also carried a β-lactamase on the same plasmid, indicating that they may be transferred simultaneously. 5. All *qnr*-positive isolates identified as *Aeromonas* species harboured the same *qnrS* allele, namely the *qnrS2*.
Laht et al. (2014)	1. Investigation of the role of three WWTP in the distribution of ARGs in receiving environments.	Primary treatment, CAS treatment and tertiary treatment.	Helsinki, Tallinn and Tartu, Finland and Estonia.	-	**Physicochemical characteristics:** BOD$_7$, TSS, total phosphorus, total nitrogen	**ARGs:** *sul1, sul2, tetM, tetC, blashv-34, blactx-m-32, blaoxa-58*	16S rRNA	qPCR.	1. *sul1, sul2,* and *tetM* were detected in all samples. 2. Statistically significant differences between the inflow and effluent were detected in only four cases. 3. Effluent values for *blaOXA-58* and *tetC* decreased in the two larger plants while *tetM* decreased in the medium-sized plant. 4. Only *blaSHV-34* increased in the effluent from the medium-sized plant. In all other cases the purification process caused no significant change in the relative abundance of ARGs, while the raw abundances fell by several orders of magnitude. 5. Standard water quality variables (BOD$_7$, total phosphorus and nitrogen, etc.) were weakly related or unrelated to the relative abundance of ARGs.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
all: **IMPACTS ON THE RECEIVING ENVIRONMENT OF BIOLOGICAL WASTEWATER TREATMENT PROCESSES**									
Czekalski et al. (2014)	1. Investigate the spatial distribution of different ARGs (*sul1, sul2, tetB, tet(M), tetW* and *qnrA*) in freshwater lake sediments in the vicinity of a point source of treated wastewater.	Chemical phosphate removal process and an aerobic biological treatment, followed by clarification. Only part of the water receives a more elaborate biological denitrification treatment. Disinfection is not performed.	Lausanne, Switzerland.	Bacterial community similarity analysis.	**Metals:** Hg.	**ARGs:** *sul1, sul2, tetB, tetM, tetW.*		Automated ribosomal intergenic spacer analysis (ARISA)-PCR, sequencing, qPCR.	1. Total and relative abundance of ARGs in close proximity of the sewage discharge point were up to 200-fold above levels measured at a remote reference site (center of the lake) and decreased exponentially with distance. 2. Similar trends were observed in the spatial distribution of different ARGs, whereas distributions of ARGs and THg were only moderately correlated, indicating differences in the transport and fate of these pollutants or additional sources of ARG contamination. 3. The spatial pattern of ARGs contamination and supporting data suggest that deposition of particle-associated wastewater bacteria rather than co-selection by, for example, heavy metals was the main cause of sediment ARGs contamination.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
					IMPACTS ON THE RECEIVING ENVIRONMENT OF BIOLOGICAL WASTEWATER TREATMENT PROCESSES				
Marti et al. (2013)	1. Determination of the prevalence of ARGs; 2. Examination of how bacterial communities from biofilms and sediments respond to the discharge of WWTP effluents in receiving rivers; 3. Determination of the contribution of wastewater discharge to antibiotic resistance in the downstream river samples.	Primary treatment and secondary CAS treatment.	Ter River,Catalonia, Spain.	Metagenomic analysis.	Clarithromycin, trimethoprim, metronidazole, sulfamethoxazole, ciprofloxacin	**ARGs:** *blaTEM, blaCTX-M, blaSHV, qnrA, qnrB, qnrS, tetO, tetW, sul1, sul2, ermB*	16S rRNA	qPCR, 16S rRNA gene-based pyrosequencing	1. *blaTEM, blaCTX-M, blaSHV, qnrS, tetO, tetW, sul1, sul2, ermB* were detected in all biofilm and sediment samples. 2. There was an increase in the relative ARGs abundance in biofilm samples downstream of the WWTP. 3. There were significant differences in the community structure between downstream and upstream environments.

2.2.1. ARB&ARGs deposit in the sediments of aquatic receiving environments

The ARGs concentration of two WWTP effluents that enter a single river catchment was examined by Rowe et al. (2016), with the use of metagenomic analyses. The results revealed an array of ARGs, MGE and pathogenic bacteria, which was more rich and diverse compared to a background sample taken from a non-WWTP effluent exposed site of the river. A two-fold increase in ARGs was observed in the WWTP effluent-influenced river samples, compared to the background samples, highlighting the potential impact of the WWTP effluent discharge on the aquatic and benthic river microbiome. A study by Lopes et al. (2016) examined the relation of the presence of thermotolerant antibiotic-resistant *E. coli* in a riverine receiving environment of a anaerobic fluidized bed reactor (RALF) with physicochemical effluent and sediment characteristics, including temperature, total solids (TS), volatile suspended solids (VSS) and total suspended solids (TSS). Overall, it was shown that the effluent discharge has the potential to contribute to the occurrence of ARB in the receiving body, while upstream of the discharge point 92%-100% of the *E. coli* isolates were susceptible to an array of 12 antibiotics, indicating the importance of proximity to the WWTP discharge point source as a hotspot of ARB&ARGs. This finding is supported by Proia et al. (2016), where low ARGs abundance in benthic biofilms was observed upstream of the WWTP compared to the abundance downstream of the examined WWTP discharge points, with ARGs abundance being noticeable even 1 km downstream of WWTP discharge. Similar findings were given by Marti et al. (2013), where there was an increase in relative ARGs abundance in biofilm samples downstream of the WWTP, using a metagenomic investigative approach. This result agrees with the findings of Berglund et al. (2015), according to whom there was an increase in ARGs and integrons (*intl1*) downstream of the WWTP in a receiving riverine environment. The authors attribute this increase to the accumulation of ARGs that are discharged from the WWTP, through the mechanism of deposition in river sediments. Antibiotics on the other hand, were not found in the downstream environment of a WWTP discharge point, besides fluoroquinolones, a fact that as the authors argue, demonstrates their high persistence and capacity to be retained in river sediments (Marti et al. 2014). A 200-fold increase in ARGs absolute and relative abundance compared to a reference site, was observed in the center of a lake in Switzerland by Czekalski et al. (2014). Interestingly, the relative and absolute ARGs levels decreased exponentially as the

distance from the center of the lake increased, revealing an inverse pattern between distance from an ARGs hotspot and of ARGs concentrations. Deposition of particle-associated bacteria rather than co-selection of ARG were once again the main factor for the ARG contamination and accumulation in a receiving environment.

A study by Li et al. (2015) investigated the effect of an A^2/O-UV-C process and of a triple oxidation process (OX) followed by UV-C, on the reduction of heterotrophic plate counts resistant to tetracycline and sulfamethoxazole, and on various ARGs. At the same time, the concentrations of various antibiotics were determined in the same treated effluents, and the correlation between antibiotic residues and ARB&ARGs was determined, in the treated effluent and in a receiving lake body. The results indicated an increase in the concentrations of specific antibiotics in the lake (sulfamethazine and sulfamethoxazole), possibly due to the deposition mechanisms in place. At the same time, total ARGs abundance increased by 0.1 log in the lake environment and some ARGs such as *tetC*, *intI1* and *tetA* increased, possibly due to the high input of the effluent and their deposition in sediments. Xu et al. (2015) also have similar findings, as their study demonstrated an increase in ARGs in the receiving river, while residual tetracycline concentrations were found to be strongly statistically correlated (R>0.8) to *tetB* and t*etW* abundance in both WWTP effluent and river samples examined, suggesting that the WWTP biological process (aerobic/anoxic) may play an important role in the prevalence of ARGs in effluent-receiving riverine environments. The effectiveness of the biological filter treatment as a post-secondary biological treatment process was evaluated by Laht et al. (2014). The results showed no significant changes in the ARGs relative abundance, at the same time that absolute ARGs abundances fell by several orders of magnitude.

2.2.2. WWTP effluents can alter the physicochemical and (micro)biological structure of receiving environments

In addition to the deposition phenomena of microcontaminants including ARB&ARGs, WWTP effluents have been shown to significantly alter the hydrology, physico-chemistry and biological characteristics of the receiving streams in a study by Proia et al. (2016). In this study, the effect of the discharge of WWTP effluents on a riverine receiving environment was examined, and more specifically on the prevalence of ARGs in formed biofilms. The authors have observed that microbial benthic communities found in the receiving river which were in immediate contact to the WWTP had a higher

ARGs abundance, indicating the favouring of the persistence and spread of ARGs in sediments influenced by WWTP effluents.

2.2.3. *Physiochemical WWTP effluent parameters can influence the abundance of ARB&ARGs in receiving environments*

Various biotic and abiotic factors shape the bacterial communities in a WWTP in a way that species that are resistant to specific antibiotics, increase in abundance even in the absence of any selection pressure from antibiotics. As a result, potential relationships between antibiotic concentrations and ARGs abundance may be confounded by these un-included factors that affect antibiotic resistance profiles (Bengtsson-Palme et al. 2016).

The evaluation of statistical correlations between the abundance of thermotolerant *E. coli* and the examined physicochemical parameters, namely dissolved organic matter (DOC), total solids (TS) and total suspended solids (TSS) revealed a correlation (R=0.94) between the percentage of resistant isolates among total isolates, with VSS amounts and temperature. This positive correlation indicated the effect that the DOC contained in released effluents might have on the prevalence of ARB, as a high DOC amount may promote HGT events and strengthen the antibiotic-resistant communities within total bacterial communities. Finally, this study indicated a negative correlation between TS and TSS, suggesting that a reduced TSS amount may encourage the prevalence of ARB in treated effluents and receiving body environments. This argument is supported by the study of Proia et al. (2016), where the examination of WWTP effluent and benthic environment physicochemical characteristics, showed that the increase in river flow caused by the WWTP effluents caused an increase in nutrient concentrations, also producing alterations in the microbial benthic community structural properties. The biofilm biomass increased downstream of the WWTP discharge point, being positively correlated through statistical analysis with TN and TP concentrations. Also, a high contribution of the WWTP effluent was shown to increase the abundance of ARGs (especially *ermB* and *sul*1) in the benthic samples examined, probably due to the negligible dilution of the effluent, as argued by the authors. Laht et al. (2014) however, found very weak correlations between effluent and river water physicochemical parameters (BOD$_7$, TP, TN) and the relative ARGs abundance in the examined samples, indicating the non-causative relationship between the two parameters.

2.3. Sources and carriers of ARB&ARGs

The identification and characterization of bacterial carriers of antibiotic resistance is crucial for the investigation of the most important routes of dissemination of antibiotic resistance in aquatic environments. The study and understanding of the mechanisms and patterns of transmission of ARGs between distinct environmental compartments, such as in the case of transfer from hospital to municipal WWTP, which have the capacity to ultimately contaminate surface water or soils, is vital for the mitigation of the spread of antibiotic resistance in the environment. So far, few bacterial species have been studied, i.e. *E. coli* and enterococci, while other bacteria found in different environments may be important actors in carrying antibiotic resistance. The most relevant studies regarding the examination of sources and carriers of antibiotic resistance, published between 2012 and 2017 are found in Table 3.

Table 3 - Comprehensive review of the studies examining the sources and carriers of antibiotic resistance in urban wastewater.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SOURCES AND CARRIERS OF ANTIBIOTIC RESISTANCE IN URBAN WASTEWATER									
Varela et al. (2016)	1. Assess the role of quinolone-resistant *Aeromonas* spp. on the dissemination of antibiotic resistance; 2. Elucidation of the role of hospital effluent as major reservoir or if, in contrast, *Aeromonas* spp. could be an indicator of antibiotic resistance from non-clinical sources.	Preliminary treatment where fats and bulky solids are removed followed by decantation in a primary settling tank and further direction to secondary treatment in a biological reactor responsible for the removal of organic matter, composed of an anoxic (denitrification), an aerated (nitrification) and an endogenous (phosphorous removal) zone. The final treatment consists of sand bed filtration, for the removal of suspended solids.	Northern Portugal.	Nalidixic acid, ciprofloxacin, amoxicillin, ceftazidime, streptomycin, sulfamethoxazole/trimethoprim, tetracycline, gentamycin, colistin sulfate and meropenem-resistant *Aeromonas* spp.		**ARGs:** *qnrA, qnrB, qnrC, qnrD, qnrS, aac(6')-Ib-cr, qepA, qnrVC, oqxAB, blaOXA-1-4, blaOXA-7, blaOXA-10-11, blaOXA-13-17, blaOXA-19, blaOXA-21, blaOXA-28, blaOXA-30-32 and blaOXA-34,35 and Class 1 integrons*	16S rRNA.	Amplicon sequencing, selection of isolates, conventional PCR, conjugation experiments, antibiotic susceptibility testing.	1. *Aeromonas caviae* and *Aeromonas hydrophila* species were found in all types of wáter tested being the most abundant species of the genus Aeromonas (50% and 41%, respectively); 2. *blaOXA-10-11*were identified for the first time in *Aeromonas* spp; 3. *qnrS2* were mainly detected in WWTP effluent and less in effluent originating from hospitals (51% and 3%), suggesting that its origin is not of clinical setting; 4. Members of the genus *Aeromonas* containing the genes *aac(6')-Ib-cr* and *blaOXA* are relevant tracers of antibiotic resistance dissemination in wastewater habitats, while those yielding the gene *qnrS2* allow the traceability from non-clinical sources.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SOURCES AND CARRIERS OF ANTIBIOTIC RESISTANCE IN URBAN WASTEWATER									
Vaz-Moreira et al. (2015)	1. Identify the taxonomic groups of quinolone-resistant bacteria with the highest multidrug-resistant (MDR) indices, which are released by hospital effluents and/or detected in the municipal WWTP receiving those effluents.	Preliminary treatment where fats and bulky solids are removed followed bt decantation in a primary settling tank and further direction to secondary treatment in a biological reactor responsible for the removal of organic matter, composed of an anoxic (denitrification), an aerated (nitrification) and an endogenous (phosphorous removal) zone. The final treatment consists of sand bed filtration, for the removal of suspended solids.	Northern Portugal.	Amoxicillin, ticarcillin, cephalothin, ceftazidime, meropenem, colistin sulfate, sulfamethoxazole, sulfamethoxazole/trimethoprim, ciprofloxacin, tetracycline, gentamycin, streptomycin-resistant bacteria.		**ARGs:**_qnrA, qnrB, qnrC, qnrD, qnrS,qepA, oqxAB, aac(6')-Ib-cr_	16S rRNA.	Amplicon sequencing, selection of isolates, conventional PCR, conjugation experiments, antibiotic susceptibility testing.	1. The isolates with highest MDR indices were mainly from hospital effluent and comprised ubiquitous bacterial groups of the class _Gammaproteobacteria_, of the genera _Aeromonas, Acinetobacter, Citrobacter, Enterobacter, Klebsiella_ and _Pseudomonas_, and of the class _Flavobacteria_, of the genera _Chryseobacterium_ and _Myroides_; 2. The transconjugants demonstrated acquired resistance to a maximum of five classes of antibiotics but they are non-significantly more prevalent in hospital effluent than in municipal wastewater; 3. Hospital effluent was confirmed as a source of MDR bacteria combining resistance to six or more classes of antibiotics.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SOURCES AND CARRIERS OF ANTIBIOTIC RESISTANCE IN URBAN WASTEWATER									
Rodriguez-Mozaz et al. (2015)	1. Investigation of the pollution level of a broad range of antibiotics and ARGs released from hospital and urban wastewaters; 2. Their removal through a (WWTP); 3. Their presence in the receiving river.	Hospital wastewater without any previous treatment and municipal wastewater.	Girona, Italy.		62 antibiotics.	**ARGs:**blaTEM, ermB, qnrS, sulland tetW.	Total DNA.	qPCR.	1. ARGs copy numbers of blaTEM, qnrS, ermB and sul1 were highest in hospital effluent and WWTP influent samples; 2. Although there was a reduction in ARGs copy numbers in effluents, this reduction was not uniform across ARGs; 3. Prevalence of ermB and tetW decreased after WWTP treatment but blaTEM, qnrS and sul1 prevalence increased.
Alexander et al. (2015)	1. Use a molecular approach that by-passesthe limitations of cultivation methods used in e.g. hospitals to detect and quantify genes and gene carriers of clinical significance; 2. Assess the dissemination of ARGs andopportunistic bacteria in natural populations; 3. Identify and monitor critical water systems and potential microbiological risks for human health.	Conventional three-treatment process (nitrification, denitrification, phosphorus elimination) for WWTP 1 and WWTP 3 and 4 included tertiary treatment. CAS system.	Southern region of Germany.	P. aeruginosa, Staphylococcus aureus, enterococci,Klebsiellapneumoniae	(Dehy-) erytromycin, Acetyl-sulfamethoxazol, (metabolite) chloramphenicol, chlortetracyclin, clarithromycin doxicyclin, erythromycin, metronidazol, oxytetracyclin, roxithromycin, sulfadiazin sulfadimidin, sulfamerazin sulfamethoxazol, tetracyclin trimethoprim	**ARGs:**ampC, ermB, vanA, blaVIM-1.	Total DNA.	qPCR, LC-MS.	1. The removal capacities were up to 99% for some WWTPs tested, but not in all investigated bacteria; 2. The abundance of most ARG increased in the bacterial population after conventional wastewater treatment. As a consequence, downstream surface water and also some groundwater compartments displayed high abundances of all four ARGs.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SOURCES AND CARRIERS OF ANTIBIOTIC RESISTANCE IN URBAN WASTEWATER									
Makowska et al. (2016)	1. Determine the presence of class1 integronintegrase and genes conferring resistance to tetracyclines and sulfonamides in the genomes of culturable bacteria isolated from a WWTP and the river that receives the treated wastewater.	Secondary mechanical-biological treatment. Biological treatment with activated sludge that comprises de-phosphatation followed by denitrification and nitrification.The biologically-treated WW is not disinfected.	Łężyca, Poland.	etracycline and sulfamethoxazole resistant bacteria, Culturable heterotrophic bacteria and coliform bacteria.	-	**ARGs:** *Sul1, sul2, sul3, tetA, tetB, tetC, tetD, tetE, tetG, tetK, tetL, tetM, tetO, tetS, tetAP, tetQ, tetX, tetH, tetJ, tetY, tetZ, tet30, tetBP, tetT, tetW*.	Total DNA.	Multiplex PCR.	1. The average number of tetracycline-and sulfamethoxazole-resistant bacteria as well as the total number of heterotrophic bacteria, coliforms and number of *intI1*, *sul* and *tet* gene copies per ml, *tetB* decrease after treatment; 2. The prevalence of tetracycline-and sulfonamide-resistant bacteria, of all ARGs examined and *intI1*-positive strains increased, compared to the total number of bacteria and genes, respectively; 3. The discharge of treated wastewater increased the number of *intI1*, *tet* and *sul* genes in the receiving river water both in terms of copy number per ml and relative abundance.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SOURCES AND CARRIERS OF ANTIBIOTIC RESISTANCE IN URBAN WASTEWATER									
Zhang et al. (2015)	1. Investigate the antibiotic-resistant phenotypes of cultivable heterotrophic bacteria from influent and effluent water of three WWTPs; 2. Analyse thirteen ARGs in ARB and in activated sludge from anoxic, anaerobic and aerobic compartments.	Anaerobic, anoxic and aerobic treatment processes. WWTPa has a secondary sedimentation tank and WWTPb and WWTPc contain a membrane bioreactor to replace the secondary sedimentation tank.	Wuxi, Jiangsu province, China.	Total heterotrophic bacteria and coliforms, resistant to: ampicillin, cefalozin, ceftriaxone, tetracycline, oxytetracycline, nalidixic acid, ofloxacin, gentamycin, streptomycin, sulfametoxazole, vancomycin, chloramphenicol.	-	**ARGs:** *tetA, tetB, tetE, tetO, sul1, sul2, sul3, sulA, blaSHV, blaTEM, blaCTX-M-F, StrA, StrB.*	Total DNA.	qPCR, antibiotic susceptibility testing.	1. 5% to 64% of total isolates showed resistance to >9 antibiotics and the proportion of >9-resistant bacteria to total cultivable bacteria was lower in effluent isolates compared to influent; 2. Gram-isolates dominated in influent while Gram +ve isolates dominated in effluent; 3. The ARG examined were detected in a higher prevalence in influent ARB than effluent, except for sulA and *blaCTX-M-F*; 4. The abundances of ARGs in activated sludge from two of the three plants were higher in aerobic compartments than in anoxic ones.
Alouache et al. (2014)	1. Detection and characterisation of ESBLs and associated quinolone resistance in coliform isolates recovered at the level of an urban WWTP in Algeria.	CAS process with clarifiers followed by direct discharge in the Tatareg river.	Boumerdes city, Algeria.	Total flora, total coliforms, thermotolerant coliforms and antibiotic-resistant bacteria to: Cefotaxime, ceftriaxone, aztreonam, ceftazidime, cefoxitin, cefepime, cefpirome, imipenem, kanamycin, gentamycin, sulfonamides, tetracycline, nalidixic acid, ciprofloxacin, chloramphenicol, rifampicin	**Physicochemical characteristics:** COD, BOD.	Clonal relationship between ESBL-producing isolates. **ARGs:** *blaTEM, blaCTX-M-1, blaCTX-M-2. blaCTX-M-8, blaCTX-M-9, blaCTX-M-25, blaOXA-1, qnrA, qnrB, qnrS, qnrC, qnrD, aac-(6')-Ib, qepA, gyrA, parC, intI1.*		Double-Disc Synergy Test and Enterobacterial repetitive consensus PCR (ERIC-PCR), conventional PCR, amplicon sequencing, conjugation	1. A double mutation S83L and D87N (*GyrA*) and a single mutation S80I (*ParC*) were detected in ciprofloxacin-resistant *E. coli* isolates; 2. In *K. pneumoniae*, mutations S83I (*GyrA*) + S80I (*ParC*) or single S80I mutation were detected in ciprofloxacin-resistant isolates, and no mutation was observed in ciprofloxacin-susceptible isolates; 3. The study showed a multi-drug resistance at the inflow and outflow of the WWTP, with ESBL production, plasmid-mediated quinolones

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SOURCES AND CARRIERS OF ANTIBIOTIC RESISTANCE IN URBAN WASTEWATER									
								experiments.	resistance, and mutations in topoisomerases; 4. The findings highlight the similarity of antibiotic resistance mechanisms in the clinical setting and the environment, and the role of the latter as a source of dissemination of resistance genes.
Goldstein et al. (2014)	1. Evaluation of the occurrence, concentration and antimicrobial susceptibility of VRE in four WWTP from which treated wastewater is reused at spray irrigation sites.	Screens, primary clarifier, biological treatment, secondary clarifier, sand filtration, chlorination, dechlorination.	Midwest and Mid-Atlantic region, USA.	Total enterococci and VRE resistant to: erythromycin, quinupristin, dalfopristin, vancomycin, tetracycline, gentamycin, linezolid, streptomycin, penicillin, ciprofloxacin.	-	**ARGs:***ddl, vanC1, vanC2/3*	Genomic DNA.	Conventional PCR, antimicrobial susceptibility testing.	1. VRE were detected in 27% of all wastewater samples collected and VRE represented 3% of total enterococci detected at all WWTPs; 2. More samples were VRE-positive from the Mid-Atlantic compared to the Midwest WWTP; 3. VRE concentrations decreased as treatment progressed at all WWTPs, except at Mid-Atlantic WWTP1 where there was an increase in VRE concentrations in activated sludge reactor samples; 4. VRE were not detected in chlorinated effluent, but were detected in one un-chlorinated effluent sample. All unique VRE isolates were multidrug resistant; 5. Fifty-five percent of the isolates displayed high-level aminoglycoside resistance.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SOURCES AND CARRIERS OF ANTIBIOTIC RESISTANCE IN URBAN WASTEWATER									
Korzeniewska and Harnisz (2013)	1. Investigation of the prevalence of ESBL-producing *Enterobacteriaceae* in municipal sewage, in air samples near the selected WWTP facilities and the receiving Lynariver.	Preliminary separation of solids, gravity sedimentation tanks, secondary treatment with CAS and secondary sedimentation.	Olsztyn, Poland.	ESBL-producing *Enterobacteriaceae* resistant to cefotaxime, ceftazidime and cefpodoxime.	-	**ARGs:***blaCTX, blaTEM, blaOXA, blaSHV.*	Total DNA.	PCR, conjugation assays.	1. 19.8% of the isolates were ESBL producers; 2. ESBL producers were found in 100% of WW samples, in 33.3% of river water and 23.8% of air samples in the WWTP area. 3. 10% of isolates had plasmid-mediated genes for beta-lactam resistance; 4. Genes encoding antibiotic resistance were shown to be transferrable to an *E. coli* recipient strain, which indicates a high possibility of horizontal gene transfer among strains of different genera within the sewage and environmental samples.
Koczura et al. (2012)	1. The determination of the frequency of integron presence in *Escherichia coli* isolates cultured from aWWTP, river water upstream and downstream the discharge of WWTP, and clinicalspecimens; 2. Assessment of the antimicrobial resistance of the integron-positive isolates.	Mechanical and biological wastewater treatment.	Poznań, Poland.	Amikacin, gentamycin, kanamycin, netilmicin, tobramycin, streptomycin, ampicillin, amoxicillin/clavulanic acid, piperacillin, piperacillin/tazobactam, ticarcillin, cefazolin, ceftazidime, cefoperazone, cefotaxime, cefuroxim, cephalothin, sulfamethoxazole, co-trimoxazole, trimethoprim, ciprofloxacin, norfloxacin, aztreonam, imipenem, tetracycline, chloramphenicol,	-	Class 1, 2 and 3 integrases.	Total genomic DNA.	Conventional PCR, antibiotic susceptibility testing, ERIC-PCR.	1. Integrons were detected in 11% of *E. coli* isolates in WWTP influent, in 6% of river water upstream and 14% downstream of the WWTP discharge; 2. Isolates from the downstream river, compared to those cultured from upstream river, were more frequently resistant to kanamycin, cephalotin, co-trimoxazole, trimethoprim, and fluoroquinolones; 3. Integron-bearing isolates of *E. coli,* the level of resistance to antimicrobials depends on the origin and habitat, and differs among isolates cultured from clinical, sewage and river

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
				SOURCES AND CARRIERS OF ANTIBIOTIC RESISTANCE IN URBAN WASTEWATER					
				nitrofurantoin-resistant *E. coli*.					water sample; 4. The discharge of treated wastewater may contribute to elevated frequency of integron-positive *E. coli* isolates in the river and may broaden their resistance range.

2.3.1. Ubiquitous bacterial genera may act as tools for monitoring antibiotic resistance in aquatic environments

It has been shown that *Aeromonas* genus members have the capability to carry genes that encode resistance to beta-lactams and plasmid-mediated quinolone resistance (PMQR). Harbouring these genes bears the potential of spreading antibiotic resistance via HGT (Moura et al. 2012; Varela et al. 2016). As quinolone resistance is highly capable of penetrating water and wastewater environments, vectors of PMQR such as *Aeromonas* have the potential to act as a promising monitoring tool for assessing antibiotic resistance reservoirs and dissemination of ARGs. This finding was supported by Zhang et al. (2015), where it was established the dominance of *Aeromonas* species in a WWTP influent (48.94%), while *Bacillus* was the most dominant species in the effluent (51.06%). Besides, members of the *Aeromonas* genus have an important role in the dissemination of ARGs, as they possess genomic plasticity and ecology thus being ubiquitous organisms, as they are often found in aquatic habitats that are associated with human activities, such as the WWTP (Varela et al. 2016). Such bacteria have the selective advantage of the ability of crossing from one environmental compartment to another, acquiring or transferring antibiotic resistance. At the same time, they may get well integrated into the human microbiome, thus possessing an important role of transmission of antibiotic resistance to humans (Vaz-Moreira et al. 2015).

Other genera that were identified as ubiquitous in water environments and have been acknowledged as vectors of antibiotic resistance include *Citrobacter, Enterobacter, Klebsiella, Acinetobacter* and *Pseudomonas* (belong to the class of *Gammaproteobacteria*) and *Chryseobacterium* and *Myroides* (belong to the class of *Flavobacteria*) (Vaz-Moreira et al. 2012; Marti et al. 2014).

In a study by Varela et al. (2016), it was found that the PMQR-coding gene *aac(6')-Ib-cr* was found in the majority (59%) of *Aeromonas* isolates examined from a WWTP effluent while another PMQR-coding gene *qnrS2* was found in 37% of isolates. At the same time, the isolates carrying PMQR showed a high prevalence of MDR (93%). In the same study, the most frequently detected beta-lactam gene was *blaOXA-1*, being often associated with the presence of *aac(6')-Ib-cr*. Moreover, *qnrS2* was found in 51% of urban WWTP *Aeromonas* isolates but was uncommon in hospital effluent isolates, being found only in *Aeromonas hydrophila*. Furthermore, this study found that genetically-related strains of *A. hydrophila* and *A. caviae* isolated in a hospital effluent,

only had the *qnrS2* gene when they originated in an urban WWTP, indicating a genetic relationship between the strains isolated from the two distinct environments. The existence of this relationship is supported by studies that examined the multi-locus sequence typing of *E. coli* isolates obtained from a hospital and urban WWTP effluent, finding that the strains recovered in both locations were genetically closely related (Slekovec et al. 2012; Varela et al. 2016). The HGT of ARGs from one bacterial species to another has been well documented (Alexander et al. 2015) and the mechanisms which contribute to the antibiotic resistance patterns in aquatic habitats are of major importance in configuring successful antibiotic resistance mitigation strategies in aquatic environments.

Extended-spectrum beta-lactamases (ESBL) are enzymes that hydrolyze antibiotics belonging to the beta-lactams such as penicillins, cephalosporins of the first, second and third generation and aztreonam. There are currently more than 200 genes encoding for ESBL, with the TEM and SHV families being the most prevalent ones until the 1990s, when they were replaced by *CTX-M*, *OXA* and *AmpC* families (Amador et al. 2015). The wide use of beta-lactams has urged Amador et al. (2015) to study the antibiotic resistance patterns in *Enterobacteriaceae*, caused by human activities such as household, hospital and WWTP and their contribution into the receiving environment, in Portugal. The findings of the study showed a high frequency of beta-lactam resistance (<53.5%) in all examined samples making it an important indicator of anthropogenic pollution, while the hospital effluent showed the highest resistance rates. Furthermore, among the 383 *Enterobacteriaceae* isolates of this study, 66.6% were multi-drug resistant and 55.3% were resistant to at least 3 antibiotics highlighting the importance of these bacteria as carriers of MDR to beta-lactams.

Pathogenic *E. coli* are highly adapted for carrying ARGs from external environments such as WWTP back into the human gut, as they are ubiquitous organisms which are able to live both intra- and extra-intestinally. As these pathogenic bacteria have a high potential of ARGs possession, they survive exposure to antibiotics, increasing the likelihood of ARGs transmission to the rest of the microbial community, this being inside the human gut or in natural aquatic environments. Their extra-intestinal virulence factors provide a competitive advantage for intestinal colonization increasing the hazard of their presence especially in the case they are resistant to antibiotics. As a result, these highly adapted microorganisms form ideal carriers of ARGs in various environments (Biswal et al. 2014). A study by Biswal et al. (2014) compared the impact of the conventional CAS process and physicochemical WWTP processes on ARGs

prevalence in *E. coli* isolates, while assessing the co-occurrence of ARGs with virulence genes in the obtained isolates. It was found that there was an increase after CAS treatment of multi-drug resistant *E. coli* while the same multi-drug *E. coli* decreased in prevalence after the physicochemical processes. Also, it was shown that ARG mobility through insertion sequence elements (i.e. transposons and integrons) has an important role in the acquisition of ARGs during biological treatment but not during physicochemical treatment. Tracking the patterns of presence of *E. coli* in human and environmental compartments has also been shown useful in the tracking of virulent genotypes. It was shown that the occurrence of pathogenic isolates is significantly higher in beta-lactam ARGs carrying isolates (48% of total isolates) compared to those not carrying these genes.

An important outcome of this section is the necessity for tracing of environmental genetic determinants of antibiotic resistance and relevant carriers, in different geographic locations. The determination of the geographic distribution of antibiotic resistance determinants will establish their relation to human activities or to environmental factors, which enable their spread and dissemination in aquatic environments.

2.3.2. Acquired ARGs may be important drivers for antibiotic resistance dissemination

Acquired ARGs that are located on mobile genetic elements including plasmids, transposons and integrons, are important drivers of dissemination of antibiotic resistance. As a result, wastewater-related bacteria have the capability to spread their resistance genes to environmental bacteria (Goldstein et al. 2014), through acquired ARGs and the related dissemination mechanisms. More specifically, vancomycin resistance genes were shown to transfer between enterococci strains, as well as from enterococci to other types of bacteria, including *Staphylococcus aureus.* Class 1 integrons comprise one of the major types of mobile genetic elements known to be involved in the capture, mobilization and spread of ARGs in Gram-negative bacteria. Indeed, class 1 integrons were found to be the most common in multi-resistant bacteria, and they are modular, mosaic structures which are composed of varying combinations of MGE (Partridge 2011). They are genetic platforms that are responsible for the integration and re-arrangement of resistance determinants, or gene casettes (Koczura et al. 2012). The platform of the integron possesses a DNA fragment that consists of an integrase gene belonging to the tyrosine recombinases (*Intl1*), a primary recombination

site (*attI*) and a Pc promoter that directs the transcription of the captured gene casettes. MDR dissemination has been attributed to three classes of integrons, with class 1 integrons being the most ubiquitous among ARB, thus playing an important role in the wide dissemination of ARGs (Makowska et al. 2016). As *IntI1* has been widely utilized as a HGT platform between environmental and pathogenic bacteria, its use has been proposed as a proxy of pollution for anthropogenic pollutants including antibiotic resistance, due to the fact that it exhibits rapid response rates to diverse environmental pressures (Chow et al. 2015). However, according to Karkman et al. (2016), the class 1 integron is not a suitable universal marker for ARGs enrichment in clinical and environmental settings, as this integron provides evidence of clinically originated pollution and does not give information on all origins of anthropogenic pollution.

In the study by Vaz-Moreira et al. (2015), class 1 integron genes prevailed in PMQR isolates, indicating the role of these genetic elements in the horizontal transfer of antibiotic resistance, while Makowska et al. (2016) determined the presence of integrons and ARGs conferring sulfonamide and tetracycline resistance in the genomes of cultivable heterotrophic bacteria as well as metagenomic DNA obtained from untreated and WWTP-treated wastewater, as well as in the receiving river water. The findings of this study showed the highest frequency of the *IntI1* in the WWTP effluent (19.7%) followed by untreated wastewater (16.1%) and the lowest frequency in the river water upstream of the effluent discharge point (9.3%). In addition, the relative abundance of *intI1* was higher in the effluent compared to the untreated wastewater, indicating the effect of HGT as a consequence of stress-driven selection, caused possibly by the presence of antibiotic residues, disinfectants and heavy metals which were not adequately removed during the WWTP process. This finding was accompanied by a reduction in the number of *IntI1*-positive isolates in the effluent compared to untreated wastewater. Despite this reduction in *IntI1*-positive bacteria, there was an increase in their number in the downstream river water, compared to the WWTP-upstream river water. As a result, it was deduced by this finding that the treated wastewater discharge has contributed to the elevated frequency of *intI1*-positive bacteria in the river water downstream of the WWTP.

Class 1 integrons were detected in 35 out of 40 ESBL-producing isolates in a study by Alouache et al. (2014), which were derived in a WWTP effluent, escorted with an increase of resistance in the outflow of the WWTP, which was attributed to the selective pressure and genetic exchange taking place in the activated sludge treatment tank of the WWTP, due to the highly complex and species-rich environment provided by the

sludge. In a study by Koczura et al. (2012), the antibiotic resistance of integrase-positive *E. coli* isolates was established in isolates of clinical and environmental origin, including the discharge of a WWTP and its associated upstream and downstream river discharge points. It was found that the majority of the isolated *E. coli* harboured class 1 integrons, which were multi-drug resistant due to the great diversity of the gene cassette content on the integrons. Further, it was noted that the origin of the isolates affected the resistance to specific antibiotics, with clinical isolates being more often resistant to aminoglycosides, cephalosporins and aztreonam compared to environmental isolates, possibly due to the selection imposed on these bacteria in the presence of antibiotic residues in the wastewater. Isolates downstream of the WWTP discharge point were more frequently resistant to kanamycin, cephalothin, co-trimoxazole, trimethoprim and fluoroquinolones. Isolates in upstream river samples of the WWTP were resistant to fewer antibiotics (2-7 classes) compared to downstream river samples (2-10 antimicrobial classes), indicating that the level of resistance depends on the origin and habitat of the bacteria, as it varies among isolates from clinical, sewage and river water samples.

2.4. Selective pressures and pressure conditions on ARB&ARGs during biological wastewater treatment

As WWTP are the main collecting bodies of household and hospital waste where commensal and pathogenic bacteria encounter selective agents such as antibiotics, the selection and spread of antibiotic resistance is favored. As a result, WWTP bridge the gap between anthropogenic environments such as hospitals and natural environments such as the receiving environments, thus allowing commensal and pathogenic bacteria to reach the natural aquatic habitats (Caucci et al. 2016). A review of the studies investigating the effect of various conditions, either environmental or WWTP operational conditions, is given in Table 4.

Table 4. Comprehensive review of the studies investigating the selective pressures and pressure conditions on ARB&ARGs during biological wastewater treatment.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SELECTIVE PRESSURES AND PRESSURE CONDITIONS ON ARB&ARG DURING BIOLOGICAL WASTEWATER TREATMENT									
Caucci et al. (2016)	*1.* Investigate the seasonal relationship of antibiotic prescriptions for outpatients and the abundance of ARGs in the wastewater and also hypothesize that the environmental seasonal changes will influence the microbial community composition of both the wastewater and the treated wastewater and therefore influence the antibiotic resistance gene copy numbers carried by the bacteria in the treated wastewater.	CAS Technology.	Dresden, Germany.	Bacterial community.		**ARGs**: *Sul1, sul2, tetM, qnrB, blashv-34, blactx-m-32, blaoxa-58, vanA, blakpc-3, mecA, dfrA1.*	Total DNA.	MiSeq Illumina sequencing, qPCR.	*1.* The relative ARGs levels were significantly different between seasons being higher during the autumn and winter. Probably due the higher prescription of antibiotics during colder months; *2.* No reduction in the relative abundance of examined ARGs after WW treatment, compared to relative abundance before WW treatment; *3.* *vanA* exhibited higher relative abundance in treated WW compared to influent WW; *4.* Clear differences in the dominant taxa between influent and effluent WW.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SELECTIVE PRESSURES AND PRESSURE CONDITIONS ON ARB&ARG DURING BIOLOGICAL WASTEWATER TREATMENT									
Karkman et al. (2016)	1. Perform a quantitative analysis of transposase and ARGs abundances from an UWTP in Helsinki, Finland over four seasons during one year.	Mechanical, chemical and biological tertiary treatment (biofilters). Anaerobic digestion for the activated sludge before drying. The WWTP does not have a disinfection step.	Helsinki, Finland.	-	-	285 ARGs and 9 transposase genes related to horizontal gene transfer (HGT)		WaferGen SmartChip qPCR array.	1. All transposases and 66% of all ARGs assayed were detected in the effluent and nine resistance genes were enriched in the effluent community; 2. The erythromycin resistance gene *ermB*, *Tn25* type transposase gene (*tnpA*-04) and quartenary ammonium resistance gene *qacEΔ1* were enriched in the sediment near the discharge pipes. 3. WWTP with tertiary treatment system analyzed substantially decreased the gene abundance and richness (>99% reduction).

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Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SELECTIVE PRESSURES AND PRESSURE CONDITIONS ON ARB&ARG DURING BIOLOGICAL WASTEWATER TREATMENT									
Bengtsson-Palme et al. (2016)	1. Shed light on whether antibiotics exert a direct selection pressure for ARB in WWTP; 2. Assess the alternative hypothesis that increases of ARGs could be due to selection by other potentially selective agents (metals and biocides) than the antibiotic they confer resistance to.	CAS system.	Stockholm, Uppsala and Lidingö, Sweden.	Bacterial community.	**Antibiotics:** Ciprofloxacin, clarithromycin, clindamycin, doxycycline, eryth-romycin, fluconazole, metronidazole, nitrofurantoin, norfloxacin, ofloxacin, oxytetracycline, rifampicin, roxithromycin, sulfamethoxazole, tetracycline, trimetoprim, metals and biocides.	-	Total DNA.	LC-MS/MS, Illumina HiSeq Sequencing.	1. No consistent enrichment of ARGs to any particular antibiotic class, for neither biocide nor metal resistance genes; 2. Strong shift from obligate to facultative anaerobes, from human faeces to sewage pipes, respectively; 3. The *OXA-48* gene was consistently enriched in surplus and digested sludge; 4. ARG abundance was not reduced throughout the WWTP in the same extent as faecal bacteria.
Amador et al. (2014)	1. The assessment of the pattern of AR contribution of some anthropological activities: household, hospital and WWTP to the treated wastewater quality released into the Mondego river in the Coimbra metropolitan area, Portugal.	Preliminary treatment, primary clarification, trickling filter biological treatment and secondary clarification.	Coimbra, Portugal.	Amoxicillin/clavulanic acid, cefpirome, aztreonam,cefoxitin, imipenem,meropenem, chloramphenicol, gentamycin, ciprofloxacin, ceftazidime, cefotaxime, trimethoprim/sulfameth oxazole, tetracycline-resistant *Enterobacteriaceae.*	**Physicochemical parameters:** Temperature, pH, TSS, BOD, turbidity, COD	**ARGs:** *blaTEM, blaSHV, blaOXA, blaCTX-M, blaCC, blaFOX, blaMOX, blaDHA, blaCIT, blaEBC*	Total DNA.	Conventional PCR, antimicrobial susceptibility testing, membrane filtration method.	1. Results revealed high frequency of resistance to the beta-lactam group, cefoxitin (53.5%), amoxicillin/clavulanic acid combination (43.5%), cefotaxime (22.7%), aztreonam (21.3) cefpirome (19.2%), ceftazidime (16.2%) and to the non beta-lactam group, trimethoprim/sulfamethoxazole (21.1%), tetracycline (18.2%), followed by ciprofloxacin (14.1%); 2. The hospital effluent showed the higher rates of resistance to all antibiotic, except two (chloramphenicol and gentamicin). Similarly, higher resistance rates were detected in the(WWTP effluent compared with the untreated influent; 3. Regarding the multidrug resistance, the highest incidence was recorded

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SELECTIVE PRESSURES AND PRESSURE CONDITIONS ON ARB&ARG DURING BIOLOGICAL WASTEWATER TREATMENT									
									in the hospital sewage and the lowest in the urban waste; 4. The most prevalent genes among the potential ESBL producers were *blaOXA* (33.3%), *blaTEM* (24.1%) and *blaCTX-M* (5.6%) and among the *AmpC* producers were *blaEBC* (38.9%), *blaFOX* (1.9%) and *blaCIT* (1.9%).
Kumar Biswal et al. (2014)	1. Comparison nof the impact of conventional activated sludge and physicochemical wastewater treatment processes on ARGs prevalence in *E. coli* isolates 2. Assessment of the co-occurrence of ARGs and virulence genes in these *E. coli*.	AS1 and AS2 use CAS treatment and PC1 and PC2 use physicochemical treatment.	Montreal, Canada.	*E. coli.*	**Physicochemical parameters:** pH, turbdity, COD, TSS	**ARGs:** *ARGs to aminoglycosides, beta-lactams, macrolides, phenicols, quinolones, sulfonamides, tetracyclines, trimethoprim, olaquindox*	-	Membrane filtration method, Genomic DNA labelling, DNA microarrays.	1. Changes in the abundance of ARGs in the *E. coli* population were different for the two treatment processes; 2. Activated sludge treatment did not change the prevalence of ARG-possessing *E. coli* but increased the abundance of ARGs in the *E. coli* genome while physicochemical treatment reduced both the prevalence of ARG-carrying *E. coli* as well as the frequency of ARGs in the *E. coli* genome; 3. Most *E. coli* isolates from the four treatment plants possessed ARGs of multiple antimicrobial classes, mainly aminoglycoside, beta-lactams, quinolone and tetracyclines; 4. These isolates harboured DNA insertion sequence elements including integrase and transposase; 5. A significant positive association was found between the occurrence of ARGs and virulence genotypes.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SELECTIVE PRESSURES AND PRESSURE CONDITIONS ON ARB&ARG DURING BIOLOGICAL WASTEWATER TREATMENT									
Birošová et al. (2014)	1. The analysis of the occurrence and levels of selected antibiotics and antibiotic resistant strains in two WWTP in Slovakia. 2. According to the seasonal differences in antibiotic consumption, antibiotic concentrations in influent and effluent water as well as antibiotic resistance of coliforms and streptococci in sewage sludge were compared.	Mechanical pre-treatment, CAS system, secondary clarifier.	Bratislava, Slovakia.	Ampicillin, ciprofloxacin, gentamycin, chloramphenicol, tetracycline-resistant total aerobies, coliforms and streptococci.	**Antibiotics:** 33 antibiotics.	**ARGs:** *tetA, tetB, tetE, tetO, sul1, sul2, sul3, sulA, blaSHV, blaTEM, blaCTX-M-F, StrA, StrB.*	Total DNA.	LC-hybrid mass analyzer Q-exactive (quadrupole coupled to orbitrap high resolution MS).	1. Among assessed antibiotics ciprofloxacin and clarithromycin were detected in highest concentrations in influent water; 2. Seasonal changes were observed only in one WWTP when antibiotic concentrations decreased; 3. Insufficient degradation of some macrolides, sulfonamides and trimethoprim was detected according to their higher concentrations in effluent water; 4. Contact of antibiotics in subinhibitory concentrations and sludge bacteria in WWTPs represent the base for the development of significant levels of microbial resistance; 5. Majority of coliform bacteria were found to be resistant to ampicillin and gentamicin. A significant seasonal difference was determined only in case of high-level resistance; 6. In summer samples, an increase in the strains resistant to concentrations higher than the resistance breakpoints established by EUCAST and NCCLS was observed.
Bruchmann et al. (2013)	1. Enrichment and identification of multi-resistant *P. aeruginosa* strains carrying more than six different clinically-relevant antibiotic resistances from	-	Mainz and Karlsruhe, Germany.	erythromycin, roxithromycin, sulfamethoxazole, gentamycin, ciprofloxacin, imipenem, ceftazidim, amikacin, azlocillin, piperacillin/tazobactam	Biofilm biomass.	**ARGs:** *sul1* **Other genes:** *las1, rhlR, rpoD, mexD*	Genomic DNA.	RT-PCR, conventional PCR.	1. The application of sulfamethoxazole, erythromycin, and roxithromycin induced changes in biofilm dynamics regarding biomass formation, spatial structure and specific gene expression in different *Pseudomonas aeruginosa* isolates; 2. Multiresistant strains responded to the

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experimental methods	Study findings
SELECTIVE PRESSURES AND PRESSURE CONDITIONS ON ARB&ARG DURING BIOLOGICAL WASTEWATER TREATMENT									
	hospital and municipal WW systems, from 2 German cities. 2. These isolates were further investigated for biofilm formation, fiofilm structure and gene expression of five different genes important for resistance and virulence of this pathogen, to access the influence of environmental present sub-MIC of antibiotics.			e-resistant *Pseudomonas aeruginosa*.					applied growth conditions with changes in transcriptional activity; 3. Here, sub-inhibitory concentrations of macrolides specifically upregulated expression of quorum sensing genes (*rhlR, lasI*), whereas sulfonamides and municipal wastewater, instead upregulated expression of specific resistant genes (*sul1*) and efflux pumps (*mexD*); 4. Antibiotic sensitive isolates demonstrated an overall higher transcriptionally activity, but did not show a specific gene response to the applied exogenous stimuli; 5. The presence of low concentrated antibiotics induced also phenotypical change in the biofilm architecture observed by 3D-imaging.
Chen et al. (2013)	1. Investigate the occurrence and removal of *tet* and *sul* resistance genes in 12 wastewater treatment systems with different treatment capacities and treatment processes; 2. Assess the factors that contribute to the diversity of ARGs in raw influents; 3. Exploration of the correlations between ARGs, integrons, and other environmental factors.	Anaerobic digester associated with eco-filter and constructed wetland (A), Anaerobic digester associated with multi-soil-layering system (B), Drop-aeration biofilm process associated with constructed wetland C, Multi-stage anaerobic	Hangzou, China.		-	**ARGs:** *tetM, tetO, tetQ, tetW, sul1, sul2, intl1*.	16S rDNA.	qPCR.	1. In the influents, the relative abundance of different ARGs showed significant variations among the sampling sites. In addition, significant correlations between the gene copy numbers and wastewater receiving capacity were observed; 2. Statistical analysis revealed a positive correlation between the gene copy numbers of *sull1* and *intl1*, whereas the gene numbers of *tetM* and *sull1* were strongly correlated with 16S rDNA.

Reference	Aim(s)	Treatment	Region and country	ARB phenotypes targeted	Quantification of other microcontaminants/ physicochemical parameters	Molecular target	DNA	Experime ntal methods	Study findings
SELECTIVE PRESSURES AND PRESSURE CONDITIONS ON ARB&ARG DURING BIOLOGICAL WASTEWATER TREATMENT									
		biological filter (D,G,H), anaerobic digester associated with multi-soil-layering system (E), multi-stage anaerobic biological filter associated with constructed wetland (F), Oxidation ditch (I,J), Anaerobic oxic (K) and Anaerobic anoxic oxic (L).							

2.4.1. Seasonal changes in antibiotic consumption, temperatures and precipitation may have an effect on ARB&ARGs abundance during and after biological wastewater treatment

The correlation of the consumption of antibiotics with the levels of antibiotic resistance worldwide is an efficient proxy for the establishment of new antibiotic-resistant infections. The relationship between antibiotic consumption with the antibiotic resistance profiles of hospital isolates have led researchers to the conclusion that strong seasonal variations in antibiotic use might lead to seasonal changes in antibiotic resistance levels in the environment. Moreover, the seasonality of antibiotic prescriptions may influence the abundance of ARB&ARGs in the wastewater. This hypothesis is supported by the study of Caucci et al. (2016), where the relative ARGs levels found in a WWTP were shown to significantly differ between seasons, with the biggest variation being observed for *sul1.* On the other hand, *blaCTX-M32, blaOXA58* and *vanA* were not strongly influenced by change of seasonal conditions, such as change in temperature and/or precipitation. However, all genes were found at a higher prevalence in the colder months of autumn and winter. The difference in ARGs levels may not solely be attributed to prescriptions of antibiotics, but also to other factors, such as temperature or precipitation of suspended solids in the sewage pipes, which may alter the survival of specific bacterial species that harbor ARGs. Another interesting finding of this study was that despite the fact that there is a strong influence of seasonality on the bacterial community in the examined samples, there was no statistical relationship between the community shift and the changes in ARGs prevalence, suggesting that ARGs are widely distributed among the identified bacterial genera, a phenomenon possibly caused by HGT mechanisms taking place during the biological wastewater treatment. In another study by Karkman et al. (2016), it was shown that there are no significant differences in ARG abundance during the different seasons in one year in a Helsinki WWTP, indicating the stability of the WWTP process accompanied by stability in wastewater temperatures inside the WWTP, throughout the year. After a metagenomic examination of the influent and effluent of the same WWTP, it was found that 60 ARGs could be found in all steps of the WWTP process (influent, activated sludge, effluent).

2.4.2. Biological process operational parameters can affect the dissemination and spread of ARB&ARGs

Operational parameters may influence the dissemination and spread of ARB&ARGs both inside the WWTP and ultimately in the receiving environments. The WWTP operational parameters such as hydraulic and solid retention times (HRT and SRT) could significantly affect genetic exchange dynamics, so these parameters have a significant potential of impacting ARGs distribution in *E. coli* passing through the WWTP system. In a study by Biswal et al. (2014) comparing ARGs prevalence during and after a CAS treatment, it was found that there is a low abundance of *E. coli* in the CAS reactor due to a low abundance in bacterial communities found inside the reactor. In addition, it is suggested in this study that the main mechanism of reduction of *E. coli* is adsorption onto solids and its grazing by protozoa, leading to the conclusion that *E. Coli* found in the biologically-treated effluent were brought in by the influent flow or were multiplied as planktonic liquor inside the CAS reactor. As a result, the HRT of *E. coli* that was released in the effluent was found to be 2.3 hours and 18 hours in the CAS reactor, allowing enough time for the incoming *E. Coli* bacteria to multiply, and thus allow ARGs spread and dissemination across the WWTP process.

Furthermore, a study aiming to compare the fate of ARGs during different biological WWTP processes using a metagenomic approach by Christgen et al. (2015), showed that the upflow anaerobic sludge blanket reactor (UASB) followed by a second-stage aerobic unit and the anaerobic hybrid reactor (AHR) followed by a second-stage aerobic unit to form two distinct anaerobic-aerobic sequence (AAS) systems, achieved a high reduction of beta-lactam, aminoglycoside and tetracycline resistance genes compared to the, indicating the high capacity of the anaerobic followed by the oxygenated system to remove ARGs, compared to each process alone. However, sulfonamide and chloramphenicol resistance genes were not effectively removed by the AAS systems, despite the fact that the phenotypic prevalence of ARGs moved from target specific ARGs to MDR, from influent to effluent. Besides, it was concluded in this study that aerobic processes, making use of oxygenated environments, perform better in the reduction of ARGs. As a result, a well-designed AAS system may provide equivalent treatment performances to aerobic systems, with the added advantage of ARGs reduction with less energy.

Likewise, the role of oxygen in different biological wastewater treatment process steps for the reduction of ARGs was discussed by Du et al. (2015). In this study, an anaerobic/anoxic/oxic treatment followed by MBR (A^2/O-MBR) was employed to treat

urban wastewater. It was found in this study that the absolute concentrations of ARGs were found to decrease in the anaerobic and anoxic treatment compartments. However, their concentrations increased in the aerobic effluent, followed by a sharp decline in the MBR effluent. The reason for the high difference in ARGs reduction efficiencies may lie in the presence of aerobic pathogenic microorganisms in the aerobic treatment, which may proliferate ARGs, a phenomenon that may take place at a lesser degree in anaerobic and/or anoxic treatment steps. Furthermore, the removal of solids from the wastewater which are above the pore size, by the MBR was shown to have an important role in the decrease of ARGs, due to the physical membrane separation step, holding all solids above the membrane pore size, in the reactor. As a result, the role of oxygen in the decrease of ARGs in biologically treated effluents was argued by Tao et al. (2016), where it was shown that dissolved oxygen may play an important role in the ARGs profile in a wastewater matrix; this was conferred as the aerobic sludge ARGs profile was different than the anaerobic sludge profile, despite the fact that pharmaceutical WWTP sludge was similar to urban WWTP sludge. Furthermore, the function of temperature and COD in the occurrence of ARB&ARGs was examined by Novo et al. (2013), showing that there is a role of temperature and COD levels to be played on the structural variations found in bacterial communities of raw and treated wastewater.

2.4.3. The presence of antibiotic residues may select for antibiotic resistance determinants in aquatic environments

One of the main drivers of antibiotic resistance development is the selective pressure of antimicrobial substances even in minimum inhibitory concentrations (MIC) on bacteria (Alexander et al. 2015). Even at MIC, antibiotics have the ability of inducing a variety of genetic responses, which consequently lead to adaptation; mutations and HGT. The selective pressure is a critical point to the emergence of novel resistance determinants as it is a necessity for fixation of mutations or gene transfer events in bacterial populations. The selective pressure exerted onto bacteria in the presence of antibiotics increases the rate of mutation, induces transposon activity, recombination and DNA mobilization (Bengtsson-Palme and Larsson 2015). The exerted pressure not only inhibits the survival of antibiotic-susceptible bacteria, but also increases the concentrations of ARB through the selection of mutations and promotion of HGT between bacteria found in proximity and increases the number of donors of resistance factors, increasing HGT opportunities (Zhang et al. 2015).

Quinolone antibiotics such as ciprofloxacin have been shown to induce the presence of multi-drug resistant phenotypes in aquatic bacteria, due to the role of these antibiotics as selectors, due to their persistence and immobility in WWTP and hospital effluents, sewage sludge and rivers. This findings support the hypothesis that ciprofloxacin-resistant bacteria may be important targets of selective pressure, as ciprofloxacin resistance is often associated to resistance to other antibiotics.

Selective pressure in the pressure of antibiotics may also lead to the selection of ubiquitous bacteria in the form of MDR. These groups of bacteria may primarily exist in aquatic environments and are well suited to survival in different environmental compartments, including urban WWTP effluents, rivers, hospital effluents and the human microbiome. As a result, such bacterial groups including *Klebsiella*, *Pseudomonas*, *Acinetobacter* etc. are often associated with healthcare-associated infections (ECDC, 2013).

The microbial community may also be affected by antibiotic residues at sub-MIC, through changes in biofilm behavior and microbial transcription. More specifically, MIC and sub-MIC of antibiotics have the potential to reduce virulence and bacterial fitness of bacteria through the inhibition of quorum sensing, biofilm formation and reduction of bacterial surface hydrophobicity, through alterations in gene expression levels. The structure of a biofilm may be influenced by various factors, including the nutrient carbon source and surface properties on which the biofilm is created. The presence of antibiotics was also shown to exert an effect on biofilm architecture (Wozniak and Keyser, 2004). The impact of antibiotic residues found in a wastewater effluent on bacterial biofilm formation of *P. aeruginosa* was investigated by Bruchmann et al. (Bruchmann et al. 2013). *P. aeruginosa* can be found in a planktonic or a sessile form which is attached to surfaces through the formation of biofilms, and therefore creating a protected environment against antibiotics, surviving even in the harshest conditions. It was found in this study that sub-MIC concentrations down to 1/100000 of the MIC of erythromycin, sulfamethoxazole and roxithromycin, had the potential to influence the biofilm formations and biomass. Even at such low environmentally-relevant concentrations, the stress response of the biofilms formed by *P. aeruginosa* was concentration and strain-dependent. Isolates that were sensitive to the antibiotics, in the presence of MIC of antibiotics demonstrated a higher transcriptional activity but were not able to alter specific gene expression in order to adapt to their new environment, while at the same time higher energy consumption was shown in those bacteria. Thus, this gives a selective advantage to ARB that are fitter and better adaptable, due to

lower energy demands compared to the sensitive bacteria. Finally, this study confirmed the up-regulation of quorum sensing genes that are related to virulence, as well as the increase in biofilm biomass, as an emergency response to the presence of antibiotics.

The statistical correlation between the concentration of antibiotic residues and the presence of ARG was examined by Rodriguez-Mozaz et al. (2015). In this study, correlations were found between antibiotics and the genes conferring resistance to the specific antibiotic. In more detail, there was a statistically significant relationship between ciprofloxacin and the *qnrS* gene (r=0.86, p=0.001), ofloxacin and the *qnrS* gene (r=0.81, p=0.001), cefalozin and the *blaTEM* gene (r=0.84, p=0.001), cefotaxime and the *blaTEM* gene (r=0.74, p=0.002), clarithromycin and the *ermB* gene (r=0.89, p=0.001) and sulfamethoxazole and the *sul1* gene (r=0.83, p=0.001). On the other hand, no statistically significant correlation was detected between azithromycin and the *ermB* gene. The calculated relationships were supported in this study by the increase of the ARGs abundance as concentrations of antibiotics increased, supporting the hypothesis that increased antibiotic concentrations (sometimes above the MIC) may increase the selective pressure on bacteria, leading to a selective advantage of ARB.

3. Concluding remarks

It was discussed in this review that there are various findings in the available literature regarding the reduction of ARB&ARGs in treated wastewater effluents after biological treatment and the subsequent disinfection step, where applied. To add to the knowledge regarding the fate of ARB&ARGs during biological wastewater treatment, various studies have investigated the bacterial genera that are found in these wastewaters, which have the potential to be involved in the transfer and proliferation of ARGs.

Once released into natural environments, it was shown by studies that ARB&ARGs may deposit in the sediments of aquatic receiving environments. These effluents have the potential to alter the physicochemical and (micro)biological structure of receiving environments, as their composition and microbial community differs from the natural flora and fauna of the receiving environment, altering the characteristics of the receptor. In addition, the physicochemical WWTP effluent parameters have the capacity to influence the abundance of ARB&ARGs in receiving environments, as shown by recent studies.

Studies analysing the microbial community structure in raw/treated wastewater, have demonstrated that ubiquitous bacterial genera may act as tools for monitoring antibiotic resistance in aquatic environments. These bacteria may be carriers of acquired ARGs, which in turn may be important drivers for antibiotic resistance dissemination.

The parameters that have the potential to influence the fate of ARB&ARGs in a WWTP and in the receiving environment include seasonal changes in antibiotic consumption, temperaturas and precipitation levels, biological process operational parameters which in turn have the capacity to affect the dissemination and spread of ARB&ARGs and the presence of antibiotic residues which may select for antibiotic resistance determinants in aquatic environments. Of course, the conclusions drawn in this review are based on the number of studies available to support each statement. In order to harmonise current knowledge, a framework must be applied, where experimental methodologies utilised by different research groups are comparable and uniform. In this way, the comparison between studies and their associated findings is not prone to error or bias, and does not lead to false conclusions.

Finally, more investigations are needed in order to strengthen the knowledge surrounding the contribution of biological wastewater treatment processes to the spread of ARB&ARGs in the environment.

4. Acknowledgments

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