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Development of Antibiotic Resistance in Wastewater Treatment Plants

Fateme Barancheshme and Mariya Munir

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Abstract

Antibiotic resistant bacteria and antibiotic resistance genes have been of the emerging contaminant threatening human health. The overuse of antibiotics, both in human patients and, importantly, in livestock, has led to an explosion of antibiotic-resistant bacteria, both in the U.S. and around the world. The prediction from the World Health Organization (WHO) is that, if nothing changes, the future will look a lot like the past—where people die from minor injuries that become infected. One of the goals should be a long-term sustainable balance with everything in our environment, including bacteria to promote human health. Different microbial techniques have been employed to study the occurrence and spread of antibiotic resistance in the environment, preventing us from returning to a pre-antibiotic era. Dissemination of antibiotic resistance genes to the environment is an important factor causing an increased prevalence of resistant pathogens. Their spread to multidrug-resistant pathogens is one of the most emerging clinical challenges.

Keywords: antibiotic resistant genes, antibiotic resistant bacteria, wastewater, wastewater treatment plant, microbial pathogens

1. Introduction

Antibiotics have been used broadly in the last decades for disease control as well as livestock breeding. The misuse and inappropriate disposal of antibiotics can develop antibiotic resistance bacteria (ARB) and multi-drug resistant bacteria which carry one or more antibiotic resistance genes (ARGs). In this century, ARB and ARGs are known as emerging pollutants that threaten food safety and public health [1]. Antibiotic resistance has been identified as

a main public health concern by the World Health Organization [2, 3]. Antibiotics are most common strategy used in the treatment of bacterial infections, in addition, antibacterial metals are widely used to prevent bacterial attachment and to combat biofilms in hospital and food processing settings [2].

Antibiotics have been detected in sewage effluents, ground and surface water, sewage sludge, soil, and manure. Studies on the fate of antibiotics are motivated by two main concerns; first, antibiotics in the environment may contribute to the development of antibiotic resistant pathogens, second, the ecological consequences of antibiotic contamination that may enter in the human food chain. In a study conducted by Clarke and Smith on antibiotics in biosolids, norfloxacin, ofloxacin, ciprofloxacin, and doxycycline were measured in the sludge of a Swedish WWTP. Similar concentrations of ciprofloxacin and norfloxacin were also observed in sewage sludge samples from Switzerland. The concentration of these antibiotics was at the low mg kg^{-1} dry weight range and was constant during the treatment processes. The same compounds and concentration were measured in soil that was amended by biosolids. Some of the compounds, for instance, carbamazepine and sulfamethazine can be translocated from the soil into the aerial plant components by uptake mechanisms in greenhouse plants [4].

Kim and Aga [5] studied the effects of antibiotics and ARB of wastewater treatment plants (WWTPs) on ecology and human health. Their study introduced the WWTPs as a point contamination source of persistent pharmaceuticals that affect the design and operation of treatment systems, antibiotic resistance development among pathogenic bacteria, and accumulation of persistent pharmaceuticals in soil and water. Their study estimated concentrations of antibiotics in untreated municipal wastewater in the United States and showed their possible metabolites in activated sludge [5]. They concluded that the disappearance of the parent pharmaceuticals in WWTPs does not certainly mean their complete removal. The presence of pharmaceuticals in the aquatic environment has ecotoxicological effects that impact the algal community structure and shifts the food web structure of streams [5]. The potential ecological and health impacts of antibiotics in the environment were investigated using environmental risk assessment including a two-stage process; estimation of expected introductory concentration (EIC) entering the environment and predicted environmental concentration (PEC). PEC is needed if the drug has the potential to bioaccumulate in the environment [5].

Determination and characterization of pharmaceutical compounds, antibiotics in particular, has attracted attention because of their ecotoxicological effects [5–7]. Antibiotics, such as tetracycline, sulfamethoxazole, ciprofloxacin, norfloxacin, trimethoprim, and ofloxacin, are determined in high concentration in the sludge of different WWTPs. In a study directed by Martin [6] sludge from four sludge stabilization treatments including anaerobic digestion, aerobic digestion, composting and the lagoon was monitored to detect the occurrence of 22 pharmaceutically active compounds. The average concentrations of studied compounds were 179, 310 and 142 μg/kg of sludge dry matter in primary sludge, secondary sludge, and mixed sludge, respectively. Sewage sludge that is used for the land application is always treated during one or more treatment processes namely, lime stabilization, thickening, dewatering, drying, anaerobic digestion or composting processes. However, many contaminants like antibiotic compounds may not be removed efficiently [6].

There is a report on the presence of 24 pharmaceuticals in 12 municipal and 4 livestock wastewater sludge showing that 17 and 14 pharmaceuticals were presented in municipal and livestock WWTPs, respectively. Nonsteroidal anti-inflammatory drugs were dominant in municipal WWTPs ranging from 0.42 to 367 mg/kg, whereas antibiotics (43.6 to 142 mg/kg) were dominant in livestock WWTPs [7]. The wide use of antibiotics in the livestock industry resulted in resistance of antibiotics to degradation that can lead to antibiotic resistance development in the environment [7].

The concentration of 16 antibiotics was measured in sewage and activated sludge samples using high-performance liquid chromatography-tandem mass spectrometry [8]. Statistical analysis included general analysis (averages and standard errors), cluster analysis, and correlation and regression analysis (Pearson analysis). Their study found significant correlations between the relative abundance of ARGs and the corresponding residual antibiotic concentrations and heavy metals in the effluents of WWTPs and pre-treatment units for the antibiotics or metals are suggested. Further studies are essential to prove the causation of the results of this study [8].

2. Occurrence of antibiotic resistance in WWTPs

High concentration of antibiotics and their associated ARB and ARGs in the effluent of WWTPs enter the environment through WWTPs discharges to rivers, wastewater reuse, irrigation and amending the soil by biosolids make. Antibiotic resistance genes can persist in the environment even when there is no antibiotic pressure.

Du et al. [9] studied ARGs including *tet(X), tet(W), tet(G), sul(1)*, and *intI(1)* in the influent and effluent of different units of a municipal WWTP. The studied plant possessed the anaerobic/anoxic/aerobic membrane bioreactors (MBR). The decrease of ARGs in anaerobic and anoxic units followed by an increase of ARGs in aerobic units and then decline of ARGs in MBR units was reported in this study [9]. Anaerobic and anoxic treatments methods were more effective than aerobic treatment methods at removing ARGs. Because microorganisms have lower bioactivity under anaerobic condition and the propagation of resistance genes are inhibited [9]. Furthermore, a significant positive correlation was observed between ARGs and 16S rDNA in the wastewater treatment process [9].

Wang et al. [10] conducted a study to explore the concentration of five tetracyclines, four sulfonamides, and six fluoroquinolones in the rhizosphere soil that was irrigated by reclaimed wastewater for a long time. The total concentration of tetracycline was in the range of 12.7–145.2 μg kg^{-1} while no sulfonamide was found in samples. Fluoroquinolones were randomly detected in soils and their highest total concentration was 79.2 μg kg^{-1}. Based on the results of this study, soils that are irrigated by reclaimed wastewater accumulate antibiotics in several folds higher concentrations compared to the antibiotic concentration in the wastewater [10].

Wang and his research group studied soils of six public parks which were irrigated by the reclaimed wastewater. There was no antibiotic pressure but sulfonamide resistance genes (*sul(1)* and *sul(2)*) persisted in the soil. This result indicated that ARGs are more permanent

Treatment Method	Target	Biosolid (copies/mL)	Effluent (copies/mL)	Reference
Activated sludge	tetO, tetW, sul1	1.00×10^8–1.78×10^9	9.12×10^5–1.05×10^6	[12]
Activated sludge	tetC	3.09×10^8–9.33×10^8	ND* – 1.32×10^4	[13]
chlorination	tetA	1.23×10^8–1.29×10^9	ND – 2.14×10^4	
Activated sludge and chlorination and UV	tetQ	2.51×10^8–10^9	6.31×10^3–1.58×10^6	[14]
	tetG	3.16×10^8–1.58×10^9	1.58×10^4–7.94×10^5	
Activated sludge and chlorination	tetO	9.7×10^4	2.5×10^2	[15]
	tetQ	8.7×10^4	1.6×10^2	
	tetW	1.8×10^5	4.4×10^2	
	tetH	5.6×10^4	1.6×10^1	
	tetZ	2.2×10^5	5.5×10^3	
Different WWTPs	tetW	9.53×10^8	—	[16]
	tetO	3.15×10^8		
	sul1	6.04×10^8		
Conventional	tetW	2.34×10^5–2.51×10^7	ND – 4.27×10^3	[17]
	tetO	6.31×10^6–1.74×10^9	ND – 9.12×10^3	
	sul1	5.62×10^6–2.51×10^9	2.34×10^4–5.62×10^6	

*ND = nondetectable.

Table 1. Results of studies on ARGs concentrations (tetracycline and sulfonamide) in WWTPs.

rather than antibiotics [10]. Based on the results of a study on removal of ARB and ARGs from urban wastewater, the abundance of 16S rRNA, $intI1$, $sul(1)$, $qnrS$, bla_{CTX-M}, and bla_{TEM} was increased to pre-treatment amount after 3 days of storage of treated wastewater [11]. Hence, it is important to find effective processes to prevent bacterial reactivation before discharge or reuse of wastewater. **Table 1** is reporting concentrations of ARGs in samples from biosolid and effluent of different WWTPs.

2.1. Effect of metal on ARGs development at WWTPs

The world is getting progressively more industrialized and urbanized which leads to elevation of heavy metals concentrations into the environment. Human activities such as mining, waste disposal, and corrosion of metals introduce more metal contaminations into the environment [18]. Population growth and industrial development have resulted in the increase in the discharge of industrial effluents in the environment. The effluent contains antibiotics and heavy metals which can trigger antibiotic- and heavy metal- resistance. ARB and heavy metal resistant bacteria and their associated genes are a public health concern.

Municipal wastewater is a hotspot for emerging contaminants namely antibiotics, heavy metals, ARGs, and heavy metal resistance genes (HMRGs). There are bacteria like *Escherichia coli* and *Salmonella* that are resistant to multiple antibiotics and heavy metal [19]. There is some

experimental evidence showing a relation between the acquisition of HMRGs and ARGs by the mechanism of co-selection [20, 21].

Genetic co-selection of resistance genes occurs when in the presence of a stress, the selection of the associated resistance gene results in the persistence of additional resistance genes [20, 22]. Co-selection happens even without a straight effect of their specific stressors. Antibiotics and metals, as sources of environmental stresses, can affect bacterial antibiotic susceptibility and heavy metal resistance promotion. Regularly the presence of mobile genetic elements (MGEs) carrying multiple resistant genes results in co-selection [20, 23]. The molecular mechanisms behind the development of heavy metal resistance are almost similar to mechanisms which explain antibiotic resistance like efflux, by which MGEs transfer genes [23].

There are several studies investigating the common structural and functional characteristics of antibiotic-resistance and metal-resistance systems. Antibiotics namely, chloramphenicol, ciprofloxacin, coumermycin, rifampicin, tetracycline, and trimethoprim and also metals like As, Cu, Zn, Mn, Co, Ag, Hg, Cd, and Ni have been studied [21].

In another study, low total metal levels correlate with ARG abundance in soils, implying that low metal levels may co-select for antibiotic resistance [24]. In this study, the abundance of 11 ARGs was quantified by quantitative polymerase chain reaction (qPCR) assay and compared with the metal levels in the selected soils. Copper, chromium, nickel, lead, and iron significantly correlated with the abundance of ARGs [24].

Icgen and Yilmaz [19] conducted a research on the Kızılırmak River which receives industrial discharges to study co-occurrence of heavy metal and antibiotic resistance in bacteria. Twenty-four isolates were found resistant to both heavy metal and antibiotics. Resistance to heavy metals involving lead, tin, nickel, barium, aluminum, strontium, silver and lithium ranged from 50 to 92% and more than 50% of the isolates were resistant to cephalosporin, quinolone, sulfonamide and aminoglycoside classes of antibiotics. Therefore, the discharge of antimicrobials to surface water may result in co-selection of heavy metal- and antibiotic-resistant bacteria [19]. The level of heavy metal in the river varied directly with changes in industrial discharge and rainfall. The relation between heavy metal exposure level and metal- and antibiotic-resistance was not clarified.

Antibacterial properties of heavy metals may contribute to development of resistance. Antibacterial properties of nine pure metals including titanium, cobalt, nickel, copper, zinc, zirconium, molybdenum, tin, and lead have been studied using two bacterial strains, Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* [25]. Based on the results, the antibacterial properties of various metals were different and among the tested metals, titanium and tin did not exhibit antibacterial properties [25]. Among the nine mentioned metals, copper and zinc are common metals in WWTPs [26–28] which are in contact with ARGs and HMRGs. Following paragraphs explain ARGs and HMRGs correlation in detail.

2.1.1. Resistance mechanism acting on both metals and antibiotics

High concentrations of anthropogenic metal contamination in the environment can apply co-selection pressure and result in antibiotic-resistance through genetic couplings [28].
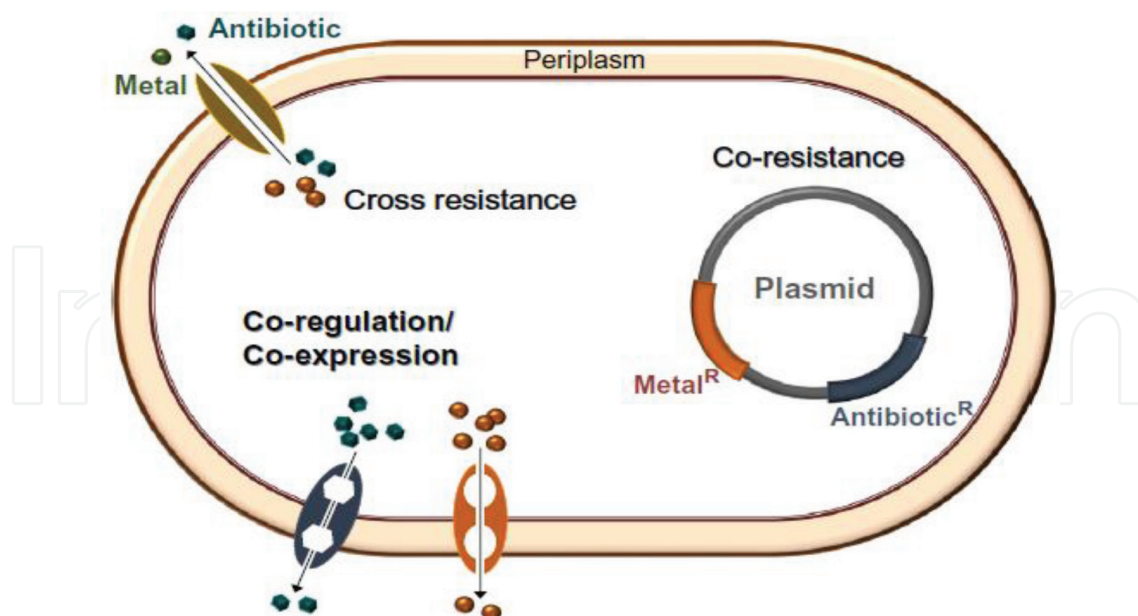
Figure 1. Mechanisms of co-occurrence of metal and antibiotic resistance [2].

Co-resistance, co-regulation, and cross-resistance are mechanisms of co-selection. As shown in **Figure 1**, a close linkage between two or more diverse resistance genes is called co-resistance and is known as a mechanism of antibiotic-metal co-selection [21, 29]. It seems that co-regulation can be a mechanism of antibiotic-resistance at which a number of transcriptional and translational responses to metal or antibiotic contact connected together to respond to both stresses [29]. Cross-resistance provides resistance to more than one antimicrobial agent like antibiotics and heavy metals [29].

There is a growing evidence of antibiotic resistance development derived from metal exposure. It seems that bacteria that are exposed to metals, like Cu and Zn, become resistant to metal and antibiotics simultaneously due to the metal selection of genetic elements that harbor both metal and antibiotic resistance genes [30]. There are many classes of antibiotics that can form complexes with metals and produced complexes can possess an enhanced or decayed antibiotic activity [30].

Di Cesare and his research group measured six ARGs (*tetA, sul(2), bla*$_{TEM}$*, bla*$_{CTX-M}$*, ermB*, and *qnrS*), two HMRGs (*czcA* and *arsB*), and the class I integron (*int1*) in different phases of three WWTPs. In their research, all the variables were classified into two groups; the first including *tetA, ermB, qnrS*, and the biotic and abiotic factors, and a second group was the genes *sul2, czcA, arsB,* and *int1*. In addition, the dynamics of *sul(2)*, HMRGs, and *int1* correlated strongly. Based on this study, there is a possible relation between heavy metal contamination as well as HMRGs and spread of ARGs [20].

2.1.2. Environmental impacts of antibiotic and heavy metal resistance genes

When a bacterial community is exposed to heavy metal as a selective pressure in WWTP the potential co-selection of resistant genes is very high [20]. Studies on genes encoding for resistance against different metals and of ARGs in plasmids and integrons demonstrated that

these genes originate from WWTPs or soils contaminated by wastewater. The class 1 integrons, specifically, are frequently associated with gene cassettes in which both HMRGs and ARGs are present and can play an important role in co-selection mechanisms [20, 25, 30].

The effluent of WWTPs contains ARGs and HMRGs which discharges to the aquatic environment, results in the spread of ARGs and increasing the risk of gene transfer to human and animal pathogens through food chains or drinking water [1, 31]. These risks require further attention and consideration while WWTP effluents are reused as irrigation water [32].

However, the idea of co-selection of ARGs and HMRGs is supported by some studies, there is a lack of data determining the exposure level to antimicrobial metals on the selection of resistance genes.

2.2. Co-occurrence of antibiotic and heavy metal resistance

The results of a study on the effect of Ni, Cu, Zn, Cd, and Pb on fate and distribution of ARGs, showed a positive correlation between individual ARG and HMRGs. This result implies that heavy metals act as selective stressors and lead to the co-selection mechanism between specific metal and antibiotic resistance [1]. In their study, the abundance of *sul(3)*, *tetA*, *tetM*, *qepA*, and *qnrA* genes had a positive correlation with Cu, Zn and Hg concentration [1].

Cu or Zn are selectors of exact bacterial populations flourishing in wastewater. Zn or Cu selected for populations of *Betaproteobacteria* and *Flavobacteria* that result in multidrug resistance against carbapenems and third-generation cephalosporins [31]. Based on the review of different studies on ARGs development in WWTP, Cu, Zn are common and in high concentration in the municipal wastewater [1, 26–28, 33–35].

Baker-Austin et al. [21] studied experimental evidence presenting a relation between HMRGs and ARGs. When the genes corresponding to resistant phenotypes are on the same genetic element (plasmid, transposon or integron) co-selection occurs, and this physical relation leads to the co-selection for other genes located on the same element [21]. The genetic traits contributed to mercury- resistance and antibiotic-resistance were established and showed that mercury- resistance was co-transferred with antibiotic resistance in a subset of mating between *Enterobacteriaceae* and recipients. There are two critical points that explain the importance of studying HMRG occurrence; co-selection mechanism and occurrence of resistance to antibacterial metals. Antibiotics namely, chloramphenicol, ciprofloxacin, coumermycin, rifampicin, tetracycline, and trimethoprim and also metals like As, Cu, Zn, Mn, Co, Ag, Hg, Cd, and Ni were studied by Baker-Austin et al. [21]. The present associations between metal contaminations and antibiotic resistance development implied the mechanisms of co-selection, including co-resistance and cross-resistance. This research group also reviewed the role of metals as a factor in co-selection and distribution of antibiotic resistance. As shown in **Table 2**, antibiotic resistance and metal resistance have common structural and functional characteristics.

Based on the literature review, there are discrepancies in our current knowledge of the dominant mechanisms of co-selection for metal- and antibiotic-resistance at the population and community level and investigation of whether metals maintain a pool of horizontally transferable antibiotic-resistance determinants.

Resistance mechanism	Metal ions	Antibiotics
Reduction in permeability	As, Cu, Zn, Mn, Co, Ag	Cip, Tet, Chlor, ß-lactams
Drug and metal alterations	As, Hg	ß-lactams, Chlor
Drug and metal efflux	Cu, Co, Zn, Cd, Ni, As	Tet, Chlor, ß-lactams
Alteration of cellular targets	Hg, Zn, Cu	Cip, ß-lactams, Trim, Rif
Drug and metal sequestration	Zn, Cd, Cu	CouA

Table 2. Shared characteristics of antibiotic and metal resistance systems [21].

3. Role of WWTP in dissemination of ARG

Freshwater resources are too limited and meeting the needs for water is challenging in the last decades as urban water shortages increase [36–38]. Based on the united nations world water development report of UNESCO in 2015, up to 70% of the fresh water, we take from rivers and groundwater is devoted to irrigation [38]. The predicted increase in the global human population to 9.7 billion in 2050 will lead to an increase in water requirement for agricultural and food production purposes [39]. Hence, the reuse of treated wastewater in agriculture seems to be a practical solution for water shortage [35]. In addition, it can help freshwater ecosystems by reducing the discharge of effluent from wastewater treatment plants (WWTPs) and preventing eutrophication and algal blooms [35].

Treated urban wastewater contains organic substances (e.g., antibiotics) and inorganic matters including pathogens, ARB and ARGs [40]. The reuse of treated wastewater may result in contamination of the environment and spread of ARB and ARGs and trigger public health concerns. One of the applications of treated wastewater is irrigation which is encouraged by governments and official organization especially because of water shortage and poverty in developing countries and urban areas [36, 41–43].

Zhang et al. [43] studied the contribution of wastewater treatment to the antibiotic resistance development of *Acinetobacter* spp. that are found in many environments, including water, soil, sewage, and food. In this study, *Acinetobacter* spp. isolates from five different sites including raw influent, second effluent, and final effluent of WWTP and upstream and downstream of the treated wastewater discharge point. This study determined the antibiotic susceptibility phenotypes using the disc-diffusion method for eight antibiotics that includes amoxicillin, chloramphenicol, ciprofloxacin, colistin, gentamicin, rifampin, sulfisoxazole, and trimethoprim. This research concluded that conventional biological treatment process in WWTPs increases the ARB population [43].

Another comprehensive study detected 140 plasmid-borne ARGs of the WWTP using polymerase chain reaction (PCR) method [44]. In this study, 192 resistance-gene-specific PCR primer pairs were designed and synthesized. Samples were collected from activated sludge and the final effluents of the WWTP. The methods included (a) isolation of plasmids from resistant bacteria, (b) selection of target reference ARGs and design of PCR primers, (c) PCR

and amplicon detection, (d) sequencing and analysis of selected resistance-gene-specific amplicons. Based on the results of this study, bacteria of the WWTP share a mobile pool of ARGs that result in genetic exchange between clinical and WWTP bacteria. The final effluent of WWTP also contained ARB that confirms that the WWTP's final effluents are disseminating antibiotic resistance in the environment [44].

Recently, Zhang et al. studied both cell-free DNA and cell-associated DNA as a source for ARGs that are related to WWTPs. The cell-free DNA is extracellular DNA that can transform into other cells, and cell-associated DNA is intercellular DNA. The 0.22 μm filter intercepts intercellular DNA and extracellular DNA (filtrates contains the extracellular DNA). In this research, four ARGs (*sul(2), tet(C), bla*$_{PSE-1}$*,* and *erm(B)*) as cell-associated and cell-free fractions were studied. The cell-associated DNA and cell-free DNA were independently extracted and ARGs copy numbers were quantified using qPCR. Based on the results of this study, cell-associated ARGs were more than ARGs fraction in the raw wastewater, however, after biological treatment, sludge settling, membrane filtration, and disinfection, cell-associated ARGs were removed considerably and cell-free ARGs removal was much lower. Therefore, the abundance ratio of cell-free ARGs to cell-associated ARGs increased. Cell-free ARGs are important pollutants from WWTPs which are potential risks to the effluent receiving environments [45].

Munir and Xagoraraki [16] quantified 18 biosolids samples from seven WWTPs using qPCR methods. The mean concentrations of *tet(W), tet(O),* and *sul(1)* in all samples of biosolids were 9.53×10^8, 3.15×10^8, and 6.04×10^8, respectively. Lime-stabilized biosolids had considerably ($p < 0.05$) lower concentrations of ARGs compared with other biosolids treatment methods. In this study, two different sites were observed for 4 months to investigate levels of ARGs (*tet(W), tet(O),* and *sul(1)*) in soils fertilized with manure or biosolids. The concentration of ARGs was higher in manure than biosolids, but surprisingly, the results showed no notable change in the concentration of ARGs in the samples of soil, since genetic diversity and natural characteristics of background soil minimized the effect of biosolids [16].

In a recent study by D'Angelo [46] on the potential risks of the presence of antibiotic in biosolid amendments, sorption and desorption of tetracycline were indicated. Their research was on four types of amendments including biosolids, poultry manure, wood chip litter, and rice hull litter at different temperatures. The sorption and desorption equilibrium constant in municipal biosolids was 20 times higher than other amendments since the concentration of bound Al^{3+} and Fe^{3+} is higher in municipal biosolids. Results showed that the sorption of tetracycline was significantly increased after treatment with alum and treatment of amendments would effectively reduce antibiotic diffusion rates [46].

The effect of treated urban wastewater irrigation on fungi diversity and soil microbial activities was studied by Alguacil and her team, in Spain. Based on this study, fungi diversity was higher in soil irrigated by fresh water, but microbial activities of soil irrigated by wastewater were much more than the soil irrigated by fresh water. Hence, wastewater not only had no negative effects on crop vitality but also developed fertility of the soil. Microbiological components are biotic factors of soil that might be altered by the increase of soil microbial biomass due to wastewater irrigation [47].

As mentioned before, WWTPs are known as sources of antibiotic resistance. Auerbach et al. [14] studied two activated sludge wastewater treatment plants and two freshwater lakes for the presence of 10 tetracycline resistance genes. Qualitative PCR and quantitative PCR methods were used to detect tetracycline resistance genes and quantify the number of tetracycline resistance gene copies per volume of sample, respectively. Their results showed that both WWTPs contain more diverse types of tetracycline resistant genes than the background natural lake water samples. They revealed that the WWTPs are a source of ARGs dissemination. *tetQ* and *tetG* in the treatment processes were attenuated, however, the UV disinfection did not reduce the ARGs [14].

Presence of specific genes encoding resistance to tetracyclines (*tetQ* [48], *tetA* [49], and *tetO* [50]), sulfonamide (*sul*1 [49] *sul*2 [50]), erythromycin (*mphB* [49]), quinolone (*qnrD* [49] and *qnrS* [50]), beta-lactams (*cepA, cfxA* [48], *bla*$_{CTX-M}$, and *bla*$_{TEM}$ [50]), erythromycin (*ermB*), methicillin (*mecA*), vancomycin (*vanA*) [50], and aminoglycoside (*aac(3)-II, aacA4, aadA, aadB, aadE, aphA1, aphA2, strA* and *strB* [51]) were analyzed and confirmed by recent studies. The results of these studies prove that WWTPs are the main source of antibiotic resistance transmission.

4. Removal of ARGs and ARB by WWTPs

The effluent of WWTPs is an important source of pollution to the nation's water resources, and 3.5 million Americans annually are getting sick after touching water they thought was safe [52]. WWTPs are hotspots for emerging contaminants namely antibiotics, heavy metals, ARGs, and HMRGs [32]. Research on the related topic has shown the proliferation of ARGs [8], the occurrence of antibiotics and ARGs and their influence on the receiving river [53], and distribution of antibiotic resistance in the effluents of WWTPs [32]. In order to limit the occurrence and spread of antibiotic resistance, treatment methods should be able to destroy ARGs in addition to inactivating pathogens [54].

Anaerobic, anoxic, and aerobic reactors were studied to treat wastewaters contaminated by high concentrations of various ARGs [9]. Aerobic and anaerobic treatment processes are low energy and environmentally friendly strategies which are mostly used to treat chemical oxygen demand (COD); moreover, they can successfully remove ARB and ARGs [55].

The aerobic treatment happens in the presence of air and microorganisms which utilize oxygen to change over organic contaminants to carbon dioxide, water, and biomass (aerobes). The anaerobic treatment forms occur in the absence of air and anaerobes microorganisms which do not require air to change over organic contaminants to methane and carbon dioxide gas and biomass [51].

Another low energy treatment alternative is anaerobic-aerobic sequence (AAS) bioreactor that reduce carbon amount as a pretreatment in an anaerobic condition and after that perform aerobic treatment [55]. Metagenomics investigations of this treatment technique demonstrated the impact of this approach on antibiotic resistance and ARGs. AAS expelled over 85% of ARGs in the influent wastewater which implies it was more proficient than aerobic and anaerobic units (83 and 62%, respectively) [55].

In another study, Munir et al. [56] investigated the occurrence and distribution of ARGs including *sul*(1), *tet*(W), and *tet*(O) and their associated bacteria in the effluent of five WWTPs to assess the efficiency of different processes. ARGs and ARB removal ranged 2.37-log to 4.56-log in activated sludge, oxidative ditch and rotatory biological contactors and 2.57-log to 7.06-log in MBR [56].

Removal of antibiotics including sulfamethazine, sulfamethoxazole, trimethoprim, and lincomycin had been studied in five different WWTPs using aerobic/anaerobic treatment methods [57]. The results of this study showed the range of −11.2% to 69.0% efficiency for different pharmaceutical compounds including sulfamethazine, sulfamethoxazole, trimethoprim, and lincomycin. The negative removal efficiency belonged to lincomycin and because of its high load in wastewater [57].

To sum it up, aerobic reactors alone are not very effective and biological treatment methods can remove antibiotics, ARB, and ARGs successfully if anaerobic and aerobic reactors operate in sequence. Despite the fact that anaerobic treatment is energy efficient and has high performance, aerobic treatment is more common in municipal WWTPs. Anaerobic treatments are often used to treat wastewater that contains high loads of organic matter like industrial wastewater and needs warm temperature (35°C). Activated sludge, which is an aerobic treatment, is studied in this project and the results will help to advance the efficiency of activated sludge bioreactors in treatment plants.

Some studies aimed to remove ARGs in raw domestic wastewater by *constructed wetlands* with different flow configurations or plant species [58]. In addition, disinfection methods including chlorination, ultraviolet (UV) irradiation and sequential UV/chlorination treatment on the inactivation of ARGs have been studied [54, 59, 60]. Recently, nanomaterials with antimicrobial activity have been offered as a novel defense against ARGs [61]. Moreover, the removal of ARGs from treated wastewater in the coagulation process was examined [62]. In one of the recent works, the effect of biochar amendment on soil ARGs was assessed and the outcomes showed that biochar is pretty operational [63].

Many diverse combinations of *nanomaterial* have proved that antimicrobial nanotechnology can be effective defenses against drug-resistant organisms, ARB, and ARGs. Two different mechanisms are probable when nanoparticles treat antibiotic resistance; the first mechanism is called Trojan Horse that develops drug-delivery characteristics. In this system, a functionalized nanomaterial is joined with antibiotics and nanomaterial enters inside cells and afterward discharge significant amounts of toxic ions [57]. In the second system, a mix of antibiotic and nanomaterials result in synergistic impacts, that means they battle ARGs independently [61]. Meanwhile, removal efficiency and mechanism of four ARGs including *tetA*, *sul2*, *ermB*, and *ampC* have been found using graphene oxide nanosheet. The removal efficiency was reported in the range of 2.88 to 3.11 logs at 300 µg/mL nanosheet solution showing the potential of graphene oxide nanosheet as an innovative and effective adsorbent for treatment of ARGs [64].

The potential for antimicrobial nanomaterials to restrict the propagation of multi-drug resistant pathogens while avoiding the generation of new nanomaterial-resistant organisms was studied by a group of researchers led by Aruguete [61]. They prepared a combination of nanomaterials functionalized with molecular antibiotics. This combination consisted of liposomes,

dendrimers, and an antibiotic that is inside of a polymer nanoparticles capsules, and inorganic nanoparticles with antibiotic molecules attached to the surfaces [61]. In this study, silver nanoparticles coated with a water-soluble polymer called polyvinylpyrrolidone were used to combat nanomaterial-resistant organisms [61]. This experiment proved that nanomaterial combinations are able to perform like an antibiotic and to be toxic to *Pseudomonas aeruginosa* bacteria which was resistant to multiple drugs [61]. The results of this study are in line with the previous reports on the silver-based polymers used as antimicrobial biomaterials for water treatment [65, 66].

Nanomaterials have been considered as a defense against multiple drug resistance because of their antimicrobial activity [59, 61, 62, 67]. Antibacterial activities of nanoparticles depend on two fundamental elements, physicochemical properties of nanoparticles and type of target bacteria. Regardless of the fact that there is a correlation in a couple of aspects of the antibacterial activity of nanoparticles, singular investigations are challenging to generalize since most of the researchers perform experiments using accessible nanoparticles and bacteria, rather than targeting particular and preferred nanoparticles or bacteria [68]. Nanoparticles which are utilized in lab-scale studies are not well-known and correlating them with physicochemical properties for full-scale production is not reliable.

A mix of nanomaterials and molecular antibiotics draws in much consideration recently, since they are effective in killing multi-drug resistant isolates of pathogenic bacterial species and combating an expansive range of ARB and ARGs [56, 69].

Nanomaterials play controversial roles in regard to antibiotic resistance; on one hand, as mentioned before, they have been considered as a defense against multiple drug resistance because of their antimicrobial activity, and on the other hand, they can encourage the development of antibiotic resistance in the environment [56, 70]. Overall, more information is needed concerning the mechanisms behind the antimicrobial activity of nanomaterials and their potential for influencing the development of resistance in environmental systems.

5. Future developments and perspectives

Antibiotic resistance development among bacteria is a challenging issue that requires improvement of next-generation treatment processes in WWTPs. The emergence of antibiotic resistance between pathogens increases the demand for effective treatment strategies. Knowledge gaps and future research needs are:

- Assessment of the effect of operating conditions (pH, free available chlorine, HRT, SRT, Biomass concentration) and environmental factors (temperature, COD, BOD, water flow on ARB and ARGs development during wastewater treatment,

- Determination of dominant mechanisms (mutation, selection, mechanisms of genetic exchange including conjugation, transduction, and transformation) of ARGs development, and

- Future studies should be done on the more extensive spectrum of ARBs and ARGs like fluoroquinolone, ertapenem, and levofloxacin resistance.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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